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Symposium on Taiwan-America Agricultural Cooperative Projects

Contributed Papers

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Welcome Opening Address

Distinguished Guests, Ladies and Gentlemen,

On behalf of the Organizing Committee of the Symposium on Taiwan-America Agricultural Cooperative Projects (STAACP), I would like to express a hearty welcome to all of you participating in the symposium. This symposium is organized by Council of Agriculture (COA), American Institute in Taiwan (AIT), Taiwan Phytopathological Society and Agricultural Research Institute of COA with the intention to promote the long-term close friendship and scientific relationship between the United States Department of Agriculture (USDA) and COA of the Executive Yuan.

The STAACP 2005 in Taipei, Taiwan, is the first attempt of the Organizing Committee aims at introducing the findings and outcomes resulted from the research projects executed through the USDA-COA Cooperative Programs in Agricultural Sciences and improving the academic collaboration between two parties. Efforts are also placed on strengthening bilateral cooperation between institutions and disciplines. This symposium has successfully called together researchers from fields of agriculture, forestry, fishery and livestock to present their works. Participants may have a chance to find their own interests from a wide range of topics included in both poster exhibition and oral sessions. The Organizing Committee will continue to encourage and push forward such collaboration and cooperation for the beneficial to both parties as well to the scientists involved.

I wish to take this opportunity to give thanks to those who are willing to dedicate their time and experience serving as moderators of the plenary and oral sessions and coordinators and secretariats of the various sections in the Executive Committee. Sincere thanks are also given to those who are present their papers and pay their contribution.

Finally, I would like to once again welcome you all and hope that this symposium very successful and fruitful to all the participants.

Chien-Yih Lin, Ph.D.
Chairman of Organizing Committee for STAACP
Director-General, Agricultural Research Institute, COA
President, Taiwan Phytopathological Society
November 15, 2005

Preface

The Symposium on Taiwan-America Agricultural Cooperative Projects (STAACP) was held in Taipei, Taiwan on 15 November 2005. This symposium was organized by Council of Agriculture (COA), American Institute in Taiwan (AIT), Taiwan Phytopathological Society and Agricultural Research Institute of COA. Its primary purpose was devoted to consolidate as well as reinforce the scientific links between the United States Department of Agriculture (USDA) and COA of the Executive Yuan.

There were four objectives of the Symposium: (1) provided a platform for the fellow experts from the United States and Taiwan to exchange innovative ideas and practical experiences; (2) introduced the scientific findings and outcomes resulted from the research projects executed through the USDA-COA Cooperative Programs in Agricultural Sciences; (3) developed integrated approaches to solve project-related problems of mutual interests; and (4) improved the academic collaboration between USDA and COA and promoted the bilateral cooperation between institutions and disciplines of both sides.

Since there is limited time left for printing the contributed papers, we have not assessed the submitted draft papers except the wording and paper type adjustment. Readers may judge for themselves the quality and wide range of topics covered in fields of agriculture, forestry, fishery and livestock. We trust that readers will enjoy and draw benefit from the contribution of the authors.

Finally, we would like to acknowledge the financial support and kind assistance from both USDA and COA. Sincere thanks are also given to those who have paid their contributions to make the symposium successful.

Acknowledgments

We are grateful for financial sponsorship from the following agencies and organization:

- Council of Agriculture, the Executive Yuan
- The United States Department of Agriculture
- Agricultural Research Institute, Council of Agriculture
- Taiwan Phytopathological Society

We also appreciate additional support from the following agencies and organizations:

- International Affairs Department, Council of Agriculture
- Agricultural Research Institute, Council of Agriculture
- Taiwan Phytopathological Society
- Food and Fertilizer Technology Center for the Asian and Pacific Region

The Symposium was planned over a year. During the period, the 11 members of the Organizing Committee (Dr. Chien-Yih Lin, Dr. Bing-Huei Chen, Dr. Grace Lih-Fang Lin, Dr. Chin-An Chang, Dr. Chang-Sheng Wang, Dr. Edward Y. Cheng, Ms. Ching-Hua Kao, Dr. Chwen-Ming Yang, Dr. Sheng-Chung Huang, Dr. Hsin-Der Shih, and Dr. Chao Chia Huang,) and the 19 members of the Executive Committee (Dr.Bing-Huei Chen, Dr. Chin-An Chang, Ms. Ching-Hua Kao, Dr. Chang-Sheng Wang, Dr. Edward Y. Cheng, Ms. Ching-Chi Tsai, Dr. Chao Chia Huang, Dr. Shwu-Jene Tsai, Mr. Jen-Ren-Chen, Dr. Chwen-Ming Yang, Ms. Yuh-Jyuan Lee, Dr. Sheng-Chung Huang, Mr.Chiu-Kai Wey, Mr. Ji-Zu Yu, Dr. Hsin-Der Shih, Mr. Jin-Hsing Huang, Mr. Yu-Bin Huang, Ms. Joanny Lee, and Mr. Kuo-Chi Lee) worked diligently to ensure the symposium was successful.

We also appreciate the efforts and hard work of Dr. Chwen-Ming Yang and Ms. Ching-Hua Kao who oversaw the publishing process of all the contributed papers.

Symposium on Taiwan-America Agricultural Cooperative Projects

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Keynote Speech

Agricultural Biotechnology Ten Years on: Lessons from the Past and Potential for the Future

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ABSTRACT

Since the first biotech crop was commercialized in 1996, today, theses crops are grown commercially by 8.25 million farmers on 200 million acres in 17 countries, and research and development is being conducted in another 45. The global commercial value of biotech crops grown in the 2003–'04 crop year is US$44 billion, 98% from five countries, US, Argentina, China, Canada and Brazil, growing one or more of biotech crops: soybeans, cotton, corn and canola. Although North America leads in research, more than half of the 63 countries engaged in biotech research, development and production are developing countries. The first generation of such crops focused largely on input agronomic traits, the next generation will focus more on value-added output traits. In the next decade some studies estimate the global value of biotech crops will increase nearly fivefold to $210 billion.

Key words: Biotechnology, Worldwide adoption, Agronomic traits, Output traits.

INTRODUCTION

Innovation is essential for sustaining and enhancing agricultural productivity. This involves new, science-based products and processes that contribute reliable methods for improving quality, productivity and environmental sustainability. Biotechnology has introduced a new dimension to such innovation, offering efficient and cost-effective means to produce a diverse array of novel, value-added products and tools. It has the potential to improve qualitative and quantitative aspects of food, feed and fiber production, reduce the dependency of agriculture on chemicals, diminish over-cultivation and erosion, and lower the cost of raw materials, all in an environmentally sustainable manner. Commercialization of the first generation of products of recombinant DNA technology was another facet in a long history of human intervention in nature for agricultural and food production purposes. As such, the same parameters of risk-based assessment should apply. Commercialization of products must be undertaken within a regulatory framework that ensures adequate protection of the consumer, the environment and alternate production systems while not stymieing innovation.

North America remains the epicenter of R&D on plant biotech, with the United States and Canada in the top five producing nations in terms of 2003-2004 commercial

biotech crop value: $2.0 billion in Canada and $27.5 billion in the United States primarily from soybeans, corn, cotton and canola. Thousands of field trials have been conducted in the two countries. From 1991 to 2004, the United States has conducted field trials, with traits being researched in 24 crops by 2004. Trials included research on fungal-resistant potatoes, peanuts, plums, bananas, rice, lettuce, salt-tolerant cucumbers, herbicide-tolerant peas, onions, tobacco and many others. Canada has produced, approved or field tested more field crops than any other country. The United States has approved in total 15 crops to date, including corn, cotton, canola, soybeans, chicory, cotton, flax, melon, papaya, potatoes, rice, squash, sugar beets, tobacco and tomatoes. In the next decade some studies estimate the global value of biotech crops will increase nearly fivefold to $210 billion.

Agricultural biotechnology has helped farmers around the world boost their productivity and grow crops in more ecologically healthy fields while allowing much more efficient use of resources. This technology allows reduced tillage, which cuts down on greenhouse gas emissions, water runoff, machinery use and soil erosion. A 2003 study of biotech crops by the National Center for Food and Agricultural Policy (NCFAP) found that eleven biotech crops planted in the United States produced an additional 5.3 billion pounds of food and fiber on the same acreage, improved farm income by $1.5 billion and reduced pesticide use by 46.6 million pounds. The NCFAP study found Roundup Ready soybeans offered several advantages to farmers, including easier weed management, less injury to crops, no restrictions on crop rotations, increase in no-till and cheaper costs. U.S. farmers using Roundup Ready soybeans saved an estimated $753 millions in 2001 due to lower herbicide costs. The broad spectrum of weeds controlled by glyphosate means that soybean growers no longer need to make as many multiple applications with combinations of herbicides. All together, the 40 case studies of 27 biotech crops showed that plant biotechnology can help Americans reap an additional 14 billion pounds of food and fiber, improve farm income by $2.5 billions and reduce pesticide use by 163 million pounds. In 2004, an additional NCFAP study demonstrated that US farmers who grew biotech crops garnered a 27 percent increase in net farm income (Sankula, 2004). Increasing yields on existing acreage reduces the pressure to convert forests and protected land into farmland. A more recent study by Brookes and Barfoot (2005) looked at the ten year impact of biotech crops since heir first large scale introduction in 1996. They showed that substantial net economic benefits at the farm level amounting to a cumulative total of $27 billions. In addition, the technology has reduced pesticide spraying by 172 million kg, reduced the environmental footprint of pesticide use by 14% and significantly reduced the release of greenhouse gas emissions from agriculture (Carbon sequestration). Biotech crops accounted for a reduction in 9.4 billion kg of carbon dioxide emissions in 2004 which is equivalent to removing five million cars from the roads.

The Food and Agriculture Organization (FAO) of the United Nations in a report issued in May 2004 found that biotechnology and genetic engineering of crops hold great promise for agriculture in developing countries. The report noted that more than

70 percent of the world's poor still live in rural areas and depend directly on agriculture for their survival. Of the eight point five million farmers who grew biotech crops in 2004, seven point six million (90%) of those were in developing countries. Agricultural research of all forms holds an important key to meeting their needs, the FAO said, and added that biotechnology can speed up conventional breeding programs and may offer solutions where conventional methods fail. That is good for growers, consumers, and anybody who cares about the environment. For example, in China, use of genetically engineered cotton eliminated the use of 156 million pounds of pesticides in 2001, an amount approximately equal to all of the pesticides used annually in California. A 2005 paper from the Royal Society suggests that intensive high-yield land-sparing farming is better for wildlife than "wildlife friendly" inefficient farming on greater acreage (Green, Royal Society 2005).

One of these modified crops, papaya is a major tropical fruit crop in the Asian region. However, production in many Asian countries is set back by the prevalence of the PRSV disease as well as post-harvest losses. The PRSV-resistant papaya, based on RNAi suppression of the coat protein expression, literally saved the $17M economy in Hawaii and is of significant importance in Taiwan and other SE Asian countries. Coat protein-based resistance is a demonstration of what is known as post-transcriptional gene silencing (PTGS). While initially it was considered a strange phenomenon limited to petunias and a few other plant species, it is now one of the hottest topics in molecular biology. RNA interference (RNAi) in animals and basal eukaryotes, quelling in fungi, and PTGS in plants are examples of a broad family of phenomena collectively called RNA silencing. In addition to protecting conventional papaya plants, by removing the virus reservoir the coat-protein (CP) protected papaya can help organic growers. There have been some claims that CP had sequences in common with a known allergen thus making them allergenic, however, this virus (with a complete in tact coat protein) has been reported on the islands since the 1940s and there have never been any reported cases ever of any allergenic reactions. Even if the CP has modified codons and was presented differently in the transgenic papaya, clinical tests have shown this makes no difference as ingested allergens are sequence specific at the amino acid level (stability) while inhaled allergens depend on protein conformation.

This system has now been applied to other species. A five year effort to combat plum pox vius disease through PTGS resistance paid off. In 1990, USDA/ Agricultural Research Service (ARS) scientists began their efforts with a papaya ringspot virus coat protein gene obtained from Dennis Gonsalves. This gene shows 70% homology to the plum pox gene and has been used to control other viruses similarly related to papaya ringspot. However, irrespective of the mechanism, it is important that resistance based on a single gene is managed well and alternate control mechanisms are introduced to reduce pressure on the development of viral resistance. Other approaches include expression of the RNA replicating enzymes of the virus, expression of satellite RNA, replicating RNA molecules that are molecular parasites of the virus or the use of protease inhibitors to interfere with processing of the viral proteins.

Scientific, civic, and religious opinion leaders from all over the world have expressed support for the value of this technology. Florence Wambugu of Kenya states that the great potential of biotechnology to increase agriculture in Africa lies in its 'packaged technology in the seed,' which ensures technology benefits without changing local cultural practices. For example over 120 million children worldwide are deficient in vitamin A. Potrykus (1999) group has engineered rice to accumulate provitamin A (b-carotene), Incorporation of this trait into rice cultivars and widespread distribution of this "packaged technology in the seed" could prevent 1 to 2 million deaths each year. She observes that in the past, many foreign donors funded high-input projects, which have not been sustainable because they have failed to address social and economic issues such as changes in cultural practice (Wambugu, 1999). In concurrence with this, Ismail Serageldin, Chairman of the CGIAR (Consultative Group on International Agricultural Research) noted that, a priori, biotechnology could contribute to food security by helping to promote sustainable agriculture centered on smallholder farmers in developing countries. For example 40% of the worlds irrigated land is no longer arable because of salinity Zhang and Blumwald (2000) have developed plants than can grow in soil that has 50 times the salt level of normal water. Similarly, a gene that produces citric acid in roots can protect plants from soils contaminated with aluminum (Lopez-Bucio J, 2000). Genes such as these can allow crops to be cultivated in hostile soils and temperatures increasing geographic range while reducing potential impact on fragile ecosystems.

The first generation of biotechnology crops focused largely on input agronomic traits, the next generation will focus more on value-added output traits. This will include identifying and isolating genes and metabolites that will make possible the enhancement of valuable traits, with some of the later compounds being produced in mass quantities for niche markets. Two of the more promising markets are nutraceuticals, or so-called "functional foods," and plants developed as bioreactors (production factories) for the commercial level production of valuable proteins and compounds, a field known as plant molecular farming (Newell- McGloughlin, 2005). Functional foods are defined as any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains. Scientific evidence is accumulating to support the role of phytochemicals and functional foods in the prevention and treatment of disease. Epidemiological research has shown a positive association between dietary intake of food components. Developing plants with improved quality traits involves overcoming a variety of technical challenges inherent to metabolic engineering programs. Both traditional plant breeding and biotechnology techniques are needed to produce plants carrying the desired quality traits. Continuing improvements in molecular and genomic technologies are contributing to the acceleration of product development. One estimate states that foods that are used for nutraceutical purposes made up 10 percent of the $503 billion total U.S. retail food market.

In addition to being a source of nutrition, plants have been a valuable wellspring of therapeutics for centuries. During the past

decade, however, intensive research has focused on expanding this source through rDNA biotechnology and essentially using plants and animals as living factories for the commercial production of vaccines, therapeutics and other valuable products such as industrial enzymes and biosynthetic feedstocks.

US Consumer attitudes also tend to be positive on the whole about agricultural biotechnology. A majority of them continue to be open to the benefits of food biotechnology, but less interested in the subject compared to other food issues, according to the latest survey conducted by the International Food Information Council (IFIC). It is notable that less than one-half of one percent of those who are avoiding something said they had avoided foods produced through biotechnology. In fact, in an International Food Information Council (IFIC) survey conducted in 2005 a clear majority of consumers (62 percent) expect food biotechnology to provide benefits for them and their families over the next five years, primarily in the form of better health/nutrition or improved food quality/taste/variety. They continue to indicate a willingness to purchase products of food biotechnology, particularly if biotechnology is used to reduce pesticides (64 percent), or improve taste and freshness (50 percent). Regarding the potential benefit of producing a more healthful oil, 41 percent state that this would have a positive impact, while 38 percent state that this would have no effect on their purchase intention. An overwhelming majority of consumers (76 percent) state that there is no information that they would like to see added to food labels, consistent with findings since 2001 when the question was first asked on the survey. Although support for the current U.S. Food and Drug Administration (FDA) policy on labeling food biotechnology has decreased over the years, more than half (55 percent) of consumers in 2005 say they support the policy, with more than a third expressing strong support. On an open-ended basis, only one percent name biotechnology as a labeling issue.

Although North America leads in research, more than half of the 63 countries engaged in biotech research, development and production are developing countries. China has emerged as a major center for biotech research. Its government has invested several hundred million dollars, ranking it second in the world in biotech research funding behind the United States.

But what of the context in which these crops are grown? Can all cropping systems co-exist in harmony? It is important to determine the relative importance of different crop production systems based on planted area, production and economic value to the region in question. The issue is what, if any, are the economic consequences of adventitious presence of material from one crop system within another based on the notion that farmers should be able to cultivate freely the crops of their choice using whichever production system works best in any given context (GM, conventional or organic). It is never a food or environmental safety issue but rather a production and marketing matter. According to Brookes and Barfoot (2004) biotech crops co-exist successfully with conventional and organic crops in North America (where, as noted, biotech crops account for the majority of acreage of important arable crops like soybeans, cotton and maize) and Spain. The market has developed practical,

proportionate and workable coexistence measures without new regulations or indeed any government intervention. Where isolated instances of adventitious presence of biotech material have been found in conventional or organic crops these have usually been caused by inadequate implementation of good coexistence practices (e.g., inefficient segregation of crops in storage and transport, nonuse of tested, certified seed). Under civil liability (i.e. tort damages) and for intellectual property infringement (except for the unauthorized StarLink), there have been no lawsuits brought by any parties for adventitious presence. Every case brought by a seed company for infringement has involved a claim that the farmer charged with infringement was an intentional infringer (i.e. adventitious presence was not the issue). And, to date, each of these cases was upheld by the courts. Indeed, all except one notable exception in North America conceded to this claim.

Within the EU, provision has been made for a *de minimis* threshold for unavoidable presence of GMOs but no actual threshold has been set. Therefore the default state of the 0.9% on labeling and traceability is the one enforced. In the US, organic products cannot be (legally) downgraded or the producer decertified by unintentional presence when all required measures and best practices are adhered to and no producer has been so impacted to date.

While biotech research and development in Europe slowed significantly following the European Union's 1998 de facto moratorium on approvals, which has since been lifted, Europe's stance on biotech crops can not prevent biotech adoption in the rest of the world and especially Asia is forging ahead. According to a study by Runge and Ryan (2004) as the EU becomes increasingly isolated, it will discourage its young scientists and technicians from pursuing European careers. If, on the other hand, the EU engages biotech in an orderly regulatory framework harmonized with the rest of the world, it will encourage a more rapid international diffusion of the technology. More nations will join the top tiers of commercial production, and emerging nations will continue to expand the sector.

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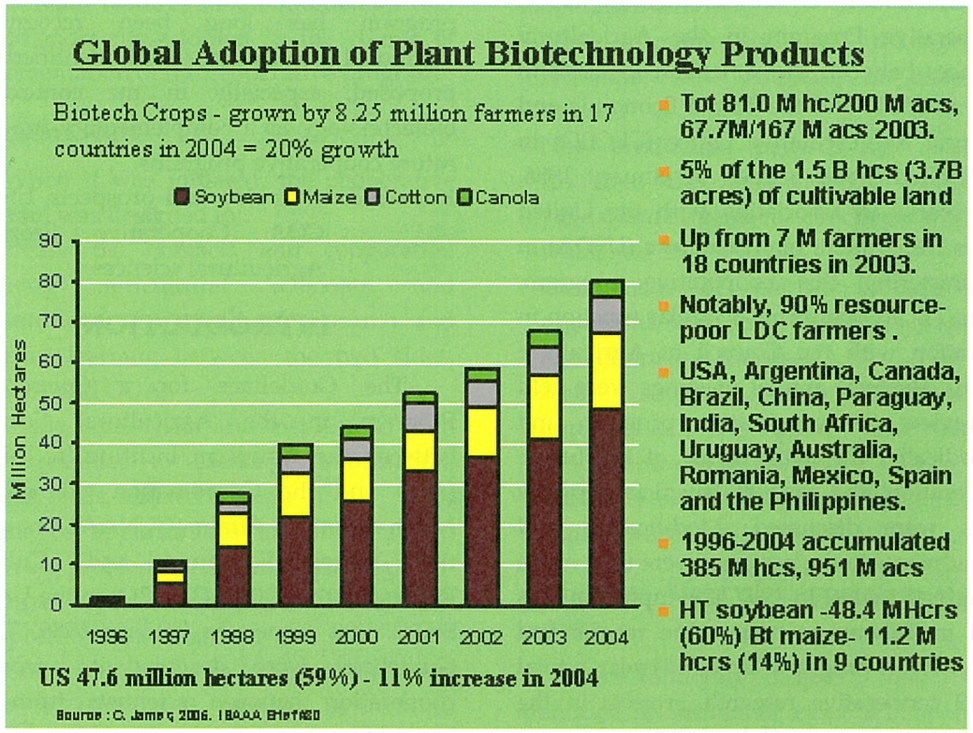

Special Report

Overview and Prospects of the USDA-COA Cooperative Program in Agricultural Sciences

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ABSTRACT

This report provides an overview of the background and history of the signing and implementation of the Guidelines for a Cooperative Program in the Agricultural Sciences between the American Institute in Taiwan (AIT) and the Taipei Economic and Cultural Representative Office (TECRO) in the United States, signed in January 1986. AIT works in association with the United States Department of Agriculture (USDA) in administering this cooperative program, while TECRO performs a similar function in affiliation with the Council of Agriculture (COA). Bilateral annual meetings were held to review the cooperative program and periodically, the main theme of the future cooperation and mutual beneficial priority areas were discussed. Modification and amendments to the program were then made on agreed issues. In 1997, a symposium was held in Taiwan to review the results and status of this program. Up to this year, a total of 99 cooperative research projects in the field of crops, animal husbandry, forestry, aquaculture, and biotechnology have been conducted. Except for a number of ongoing projects that usually cover a period of 3-4 years, most of the projects that have been duly completed received satisfactory remarks from peer reviews and were published. This report also attempts to highlight the research activities and achievements of the collaborative projects. While the importance of this cooperative program has long been recognized, additional priority areas were identified and proposed, especially in the context of biotechnology as a complementary input to future cooperative programs.

Key words: Overview and prospects, USDA-COA Cooperative Program, Agricultural sciences.

INTRODUCTION

The Guidelines for a Cooperative Program in the Agricultural Sciences between the American Institute in Taiwan (AIT) and the Coordination Council for North American Affairs (CCNAA), presently the Taipei Economic and Cultural Representative Office (TECRO) in the United States, was signed in January 1986. These Guidelines were designed to promote cooperation between scientists from the United States and Taiwan Republic of China (ROC) pursuant to the Agreement dated September 4, 1980, between the AIT and the CCNAA (hereafter referred to as TECRO). The Taiwan Relations Act (Public Law 96-8, April 10, 1979) authorized the continuation

of commercial, cultural and other relations between the people of the United States and the people of Taiwan. Such relations are conducted by or through AIT, a non-profit organization incorporated under the law of the District of Columbia, and TECRO, a non-government organization established to provide assurance and take actions on behalf of the people of Taiwan. AIT works in association with the United States Department of Agriculture (USDA) in Washington, D.C. in administering this cooperative program, while TECRO performs a similar function in affiliation with the Council of Agriculture (COA) in Taipei, Taiwan ROC. The following is an overview of the main features of the Guidelines and the amendments adopted in the course of implementation of the cooperative program.

Program Goals

When it was initiated, the cooperative program was designed to:

- Increase the contacts and cooperation among agricultural scientists and institutions of agricultural research and higher learning between the two sides, USDA and COA;
- Provide researchers and institutions with opportunities to exchange information, ideas, skills, and techniques;
- Enhance opportunities to collaborate in solving problems of common interest; and
- Utilize special facilities or research opportunities available.

Cooperation may be in any of the broad areas of basic and applied agricultural sciences, including forestry and agricultural economics. The types of cooperative activities may include the exchange of scientific information, scientific visits, seminars and workshops, and cooperative research.

Implementation and Coordination

As stipulated in the Guidelines, AIT and TECRO shall coordinate the activities agreed upon under these Guidelines, and USDA and COA shall implement them. Each side shall designate a program coordinator to be responsible for the overall coordination of cooperative activities under these Guidelines. Each side must also identify a topic coordinator.

Initial Areas of Cooperation

In 1986, the following topics were considered as priority areas for initial cooperative activities:

- New cropping and pest management techniques with emphasis on biological control;
- New production methods that conserve resources while maintaining or increasing yields, and systems for effective use of soil, water and air resources;
- Germplasm of plants, animals and organisms that have significant value in agriculture and forestry;
- Basic research and biotechnology applications for plant and animal production and protection;
- Post-harvest technology; and
- Aquaculture.

Additional Areas of Cooperation signed in 1989

The following topics have been identified as additional areas of cooperative activities, which took effect in September 1989:

- Product marketing and development;
- Forest management;
- Research and professional enhancement

involving natural reserves and wildlife management; and
- Professional enhancement and assistance involving agriculture, forestry, land preservation, land planning and urban development.

Amendments to the Areas and Scope of Cooperation were signed in 2001.

Selected priority areas for cooperation may include, but not limited to:
- Sanitary and phytosanitary technology;
- Environment, natural and marine resource management;
- Research and management of bio-technology products;
- Technology transfer and food safety;
- Horticulture and crops; and
- Animal waste management.

Review Meetings

As a usual practice, AIT and TECRO program coordinators met periodically, at time and places of their choosing, to review and adjust the program of cooperation, recommend projects to be funded, and coordinate administrative matters.

Funding

Funding of cooperative activities under these Guidelines shall be carried out on the basis of mutuality, reciprocity, and flexibility. In general, each side shall fund the cost of its participation in cooperative activities and may, as it chooses, provide full or partial support for participation in these activities by scientists of the other side. Decisions on funding for joint activities shall be made by mutual agreement. Subject to availability of funding, in particular, each side shall contribute to the cooperative activities as follows: USDA shall provide airline tickets and health insurance for participating scientists, while TECRO shall provide funding for the collaborative research activities in Taiwan, and per diem for traveling scientists from Taiwan.

Memorandum

Under the umbrella of the original Guidelines, AIT and TECRO have, in addition, signed three more memoranda as follows:
- Memorandum on Cooperation in Enhancing Commodity Situation and Outlook Reporting (February 1991)
- Memorandum on Cooperation in Forestry and Natural Resources Conservation (July 1991)
- Memorandum on Cooperation in Soil and Water Conservation (October 1992)

OVERVIEW OF PROJECT IMPLEMENTATION, 1997-PRESENT

The projects that have been implemented from the onset of the cooperative program in 1986 to date and other related information are presented in the Appendix. A Symposium for the Review of COA-USDA Agricultural Science Cooperative Program was also organized and held at the Taiwan Agricultural Research Institute (TARI), Taiwan ROC on May 5-6, 1997 for a comprehensive evaluation and assessment of the implementation of the collaborative projects. Likewise, all the abstracts of the implemented thematic subjects were compiled into a symposium proceeding. To give an overview of the progress and achievements of the collaboration projects conducted, following are brief descriptions of the research findings achieved from 1997 to date:

1. Plant Science

Pest management

Research on coordinate management and control of fruit flies

Several notorious species of fruit flies are considered as some of the most destructive agricultural pests in warm weather and tropical environment. Fruit flies have been widely placed in the watch list as one of the most important quarantine insect pests, in order to prevent their movement across national borders. They can also spread through export/import of produce and travel activities. With the increase in global trade, all countries are seeking for the most effective tools to deal with these pests – whether to control them within their vicinity or to keep out these potential invasive species.

Some useful results have been accumulated to hasten fruit fly management techniques by adopting the area-wide approach which aims to coordinate the efforts of all stakeholders, including farmers as partners. The fruit fly control research has been further improved by the team of scientists from Hawaii and Taiwan. Sixteen fruit fly control districts were established by the end of 2004. In each district, the fruit fly density data and variation of fruits and fruiting season are collected and analyzed for planning the control scheme and necessary timing adjustment. To disseminate improved technologies, the farmer education program has been extended to speed up the modernization process and provided demonstration tools for field application. The newly introduced protein bait, GF-120, has been registered and demonstrated for both fruit fly and melon fly control. In addition, the application of GIS/GPS techniques has also been incorporated to sharpen the precision of the database analysis for the coordinate control practices. The efficacy of the coordinate area-wide control approach has prompted the neighboring area of the demonstration fields to join in to form and enlarge the new areas for fruit control. Likewise, these results of the collaboration among scientists from both sides in terms of exchange of information and development new technologies were much appreciated, in addition to the methodological outcomes.

Biological control of insect pests on vegetables in pipe house

In Taiwan, crucifer crops are primarily cultivated in pipe houses. Insect pest surveys in pipe houses revealed seven species of pests, mainly aphids, thrips, mites, whiteflies, and leaf miners. Possibilities of improving current control methods used in pipe houses are to be investigated by using biological control as a viable alternative. A pest management system should also be developed to integrate the use of natural enemies, such as parasitoids and predators, with other compatible methods. Collaboration research for information on the basic biology and ecology of the parasitoids and predators have been collected for eventual use in the field or in pipe houses, to evaluate the effectiveness of these natural enemies in controlling insect pests on vegetables. In addition, further clarification is being made on the occurrence of the key insect species, their natural enemies, and their population fluctuations. Then, the species, timing, and quantity of the natural enemies released will be assessed for determining the cost-effectiveness of the biological control program and a biological control strategy will ultimately be developed.

Monitoring occurrence of late blight and its management

Late blight caused by *Phytophthora infestans* is one of the most destructive diseases of potatoes and tomatoes worldwide. Survey results revealed numerous pathogenic isolates, 113 isolates of which were found aggressive to both tomato and potato. This project will be continued to assess the occurrence and severity of late blight in tomato and potato crops through surveys in Taiwan, and to collect isolates and characterize them according to genotype, mating, and metalaxyl sensitivity.

Monitoring and development of detection kits for amaryllis plants

Many species in the amaryllis family are considered a good genetic resource and have great potential to be transformed into novel ornamental crops. However, amaryllis plants are vulnerable to many kinds of virus disease infection that threaten their production. The project succeeded in the identification and characterization of seven viruses infecting amaryllis plant species. Four of these seven viruses are new records in the literature and were named by the research team. In addition, the collaboration project has successfully developed detection kits and protocols that are essential to the development of virus-free amaryllis plant propagation system. These valuable results can be further explored for future development of commercial amaryllis ornamentals.

Potential application of Family 19 antifungal chitinases in crop management

Technological experiments were made to prepare a *Streptomyces*-derived Family 19 antifungal chitinase gene (SGS3 chi F) transgenic plant for use in disease-resistance breeding and practical crop cultivation using tomato as a model system. A preliminary survey on the expression of the gene with colloidal chitin application indicated that SGS3 chi F was among the bacterial chitinase genes preferentially expressed. A screening survey indicated that the enzyme is unique among all 16 antifungal chitinolytic *Streptomyces* tested. The serological correction and the size similarity suggested strongly that they evolved from the same biological origin. These results are very useful for future breeding of disease-resistant tomato through the transformation of bacterial chitinase genes.

Crop and nutrition

Antioxidant activities in vegetables

Increased vegetable consumption has been widely promoted in recent years because of their value to human diets by providing basic nutrients, fibers, minerals, vitamins and phytochemicals with antioxidant activities. An appropriate recommendation of particular vegetables or groups of vegetables would help to increase the consumption of antioxidants in the diets. So far, this study has evaluated 127 species of vegetables for antioxidant activities. This project has produced valuable results which can be directly used in the fields for systematic comparison of antioxidant activities in vegetables and useful information for selection of vegetables and methods for antioxidant supplements and related studies.

Antioxidant activities of tropical fruits

A total of 47 fruits were collected and analyzed for their antioxidant activities as measured by their ascorbic acid phenolic contents. The result revealed that papaya and guava had the highest ascorbic and total phenolic contents.

Floriculture

Post-harvest physiology of anthurium cut flower

Studies on the physiological responses and post-harvest handling of anthurium cut flower revealed that the cut flower is chilling sensitive. Among 11 anthurium cultivars evaluated as to their suitability for marine shipment, only five of them did not show any significant loss of quality or vase life. As to chemicals, benzylaminopurine (BA) was the most effective for most of the cultivars. However, BA induced spathe greening that lowered the cut flower quality. Meanwhile, the reduction in vase life due to chilling can be compensated for by BA treatment.

Molecular breeding of flower color

Some useful genetic materials were made using manipulation of pigment gene cloning, transformation, gene transfer, and mass propagation of transgenic plants to form new floral plants and flowers. This project aimed to develop rare yellow flower colors of chrysanthemum, caladium and anthurium using a transgenic approach. The chalcone isomerase (CHI) gene has been successfully transferred into chrysanthemum in either sense or anti-sense direction. The flowers in transgenic "Red beauty" chrysanthemum turn the color from red to yellow, indicating that the accumulation of yellow narigenin chalcone is due to the co-suppression of the endogenous CHI, which provided an alternative way for flower color to be engineered through the transformation of the CHI gene. This study was the first report on successful gene transfer to obtain yellow-color flowers and ornamental leaf plants which can be ultimately used in the development of commercial cultivars

2. Food Science

Development of biochips for detection of pathogenic bacteria in meat products

Some useful technologies have been developed by using biochips for the rapid identification of pathologic bacteria in meat products and in foods. So far, 15 oligonucleotide probes were selected and spotted on the nylon strip to determine the oligonucleotide array hybridization patterns. More than 179 strains belonging to five major pathogenic bacteria genera could be identified by using the developed oligonucleotide arrayed biochips. For such biochip, adding more oligonucleotide probes may allow the detection of more numbers of bacteria without significantly increasing the chips complexity. These results are extremely useful for the development of oligonucleotide arrayed biochips for the rapid detection of pathogenic bacteria in meat products.

3. Animal Science

Animal biotechnology

Bovine ephemeral fever

Surveys revealed that Taiwan has had eight recorded epizootics, in which the death and culls rose from about 5% in 1967 and had reached to 50% in 2002 in an important outbreak. This study evaluated the population immunity in Taiwanese dairy cows and worked on the development of a rapid diagnostic ELISA kit and new vaccines. Results showed that a single-dose vaccination induced serum neutralization antibody (SNA) level to rise and fall to a minimum after 6 months, which coincided with a focal outbreak that occurred in

September. These results provided precious information on the timing and frequency of vaccination for better prevention of the focal outbreak of the disease.

Generation of transgenic-cloned calves

This study on technological improvement for animal cloning achieved remarkable outcomes through the efforts of the collaborators from both sides, Professor Jerry Yang, the world famous expert in cow clones from the University of Connecticut, and Winston Cheng from National Taiwan University. The preliminary successes of cloned mammals, including pig, cow and dairy goat, were highly recognized in many academic papers and newspapers. The collaboration has already enhanced and provided more precious information for cloning technologies, which are and will be, inevitably intensively used for animal breeding and production.

Development of rapid diagnosis of Toxoplasma infection

Some useful results have been obtained suggesting that the recombinant fusion protein of P30 gene has antigenic epitopes with the specificity of *Toxaplasma gondii*. This recombinant protein exhibited potential to develop into a sensitive and specific antigen for rapid diagnosis of toxaplasma infection simply from blood examination.

Control of avian leucosis

There is an urgent need to develop a specific antigen detection method for Subgroup J avian leucosis virus (ALV-J) since the group-specific antigen detection is unable to distinguish exogenous from endogenous viruses. The project has successfully developed a technology for obtaining monoclonal antibodies specific to ALV-J. This antibody has been used for diagnosing ALV-J infection in chickens and the monoclonal antibody chip can be further developed for use in rapid diagnosis of avian leukosis.

Development of reliable methods for drug residue detection

In this study, a set of enzyme-linked immunosorbent assay (ELISA) kit has been developed with its detection sensitivity between 0.5 to 1 ppb. Furthermore, there is no cross reaction with major antibiotics. These results are valuable as inputs in designing new detection kits, which is projected to grow fast in the coming years.

Facility improvement and professional enhancement

The objectives of this project are to develop standard operation procedures for an animal disease diagnostic center, especially for the negative-pressured animal necropsy facility and training of animal disease diagnostic personnel. The project has formulated standard operation procedures for a negative pressured necropsy facility, laboratory tests for bovine spongiform encephalopathy, and tests for rabies. In addition, a general guideline for necropsy was provided. For training of personnel, the Animal Health Research Institute is planning to send its pathologists to enroll in pathology residency programs in the US for further advancement of pathology knowledge and competency.

4. Aquaculture

Improvement of Pacific white shrimp production

Some useful outcomes have been achieved revealed that shrimps derived from white spot disease virus (WSSD) resistant brood-stocks show a significantly higher survival rate than those of the control shrimps. It was also shown that stock obtained from rapid growth strain of Pacific white shrimp demonstrated a superior growth rate than those of regular shrimp stock. Furthermore, investigation made in clarification of the efficacy of B – Glucan produced by several fungi showed a significant contribution on the survival and growth rates of cultured Pacific white shrimps.

Water quality control of recirculation aquaculture system with algae culture

The goal of this project was to develop an integrated aquaculture model characterized by water-recirculation and low-cost production of healthy shrimp and oyster. Some useful results have been achieved showing that the integrated shrimp algae culture system was effective in controlling water quality and diseases, as well as the cost efficiency in terms of shrimp production and maintenance of the water recirculation system in operation. Oyster is not an essential component for the integrated system.

5. Forest Management

Carbon sequestration

Trees are the main sequestrator of carbon dioxide into organic carbon in the forest, making it to become part of the biomass, a most efficient natural way of carbon sequestration to alleviate global warming. Thus, proper forest management can enhance the capability of forest to sequestrate carbon and reduce accumulation of carbon dioxide in the atmosphere. The project aimed to establish a Taiwan natural forest optimal management model under the ecological management principles. Experiments conducted in a natural cypress stand showed that the maximization carbon in the steady state without any treatment could be stored 230 ton/ha. Furthermore, this stand could still store 176 ton/ha under the treatment with mild selective cutting and 13 ton/ha for the timbers after cutting for the end use. In economic performance, the stand could generate over 21 million NT$/ha for timber revenues and the soil expectation value could exceed 90 million NT$/ha. Using these models, significant benefits could be derived from the forests. These results are very useful for quantified management of natural forests, and to establish a reasonable norm for forest resources management taking both economic development and ecological balance into account.

DISCUSSION

I hope that the overview and the brief project descriptions provided everyone with a better understanding of the program, toward further enhancing the ongoing collaboration and developing new themes for our future cooperation in agricultural sciences. In this presentation, the questions that I want to pose everyone are: What impressions and inspiration could we draw from the experiences of the past cooperative program to enable us to further improve our future collaborations? How are we going to enhance and efficiently manage the collaboration that have become wider and more comprehensive to encompass various research fields in agricultural sciences, especially in the context of biotechnology and its impact on trade concerns toward coping with the current changes of the time? I envision this bilateral cooperative program to be more focused and managed more smoothly, and to better contribute to scientific and technological advancements in the years ahead. While our 20 years of

collaboration have been fruitful and rewarding in terms of formulation, identification of priority areas and implementation of the collaborative projects, greater challenges lie ahead especially in responding to structural changes in global agriculture.

Please allow me to elaborate a little bit more on the implementation and management in the early years of the cooperative program. Just imagine how much different it was then in terms of communicating among the working teams from the two sides, when east met west at the beginning. There were differences in language, status of science and technology development, calendar fiscal year in budget allocation, administrative regulations, and small things like project proposal and management, among others. But the most important thing is, we were able to manage it through and made things moving rather smoothly. I was fortunate from the beginning to be deeply involved in the cooperative program, designated as program coordinator by the COA side from 1986 until 2000. I still cherish to these days the memorable times I had working with Dr. Walker of USDA, my first counterpart coordinator for 6 years until his retirement. He was later succeeded by Dr. Bellis and Dr. Kramer-Leblanc. We worked closely in taking charge of all the coordination aspect of the program, from consultation on cooperative project identification, research proposals, monitoring project progress, and keeping track and review of the final completion report, and all these were done on an annual basis. The cooperative program has allowed researchers from numerous academic and government institutions in both the USA and Taiwan to jointly carry out 99 projects under the broad aspect of agricultural sciences, as shown in the Appendix. A total of 99 projects have been implemented classified into plant science (37), food science (9), animal science (24), aquaculture (16), and forestry (13), respectively.

This is the second workshop to be held for the purpose of reviewing the progress and achievements of the cooperative programs, the first workshop held eight years ago in 1997. Following are my comments with respect to prospects for further enhancing future collaborations under the USDA-COA cooperative program in agricultural sciences.

Enhancing Implementation and Management of the Cooperative Program

Each year during the program implementation, the program coordinator from the USDA visited Taipei, usually at the later part of the year, to join the working team in the project formulation. Summary titles and project descriptions were mailed to USDA in advance for initial assessment. At the meeting in Taipei, these project titles and summaries were reviewed and reasonable cuts were made on the budget based on deliberations by the group. By the end of February of the following year, the projects selected were submitted for peer review in Taiwan. Then the projects were sent to the US also for peer review. Usually by the end of April, mutually agreed upon projects were identified and approved, so that the approved project funding and implementation could match with the government budget fiscal calendar year starting from July 1 ending in June 30 of the subsequent year. The research projects were carried out in Taiwan, and the US collaboration counterpart scientists acted in advisory capacity making occasional visits to review or discuss the project progress. On certain occasions when Taiwan scientists needed to travel to the US, the latter side paid for the international travel.

Since the government fiscal calendar has now been changed to follow the normal calendar starting from January 1 and ending in December 31, it is advisable that the timing of bilateral meeting and project submission cycle be adjusted accordingly for a more efficient project management. It is also important that in order to ensure accountability of project monitoring, progress reports are required twice a year. Upon project completion, a final report is prepared and published. To warrant better execution, each cooperative program will be given support in duration of 3-4 year cycle but subject to annual progress review. If the problem addressed by a program is solved or is no longer a high priority after the cycle, it can be replaced by a new, much higher priority program. Toward harnessing the greatest benefit of the revised project, management system must be given better access to information about the cooperative research programs. It is suggested that a database be included as part of the Agricultural Science and Technology Information Management System which was established by the Agricultural Science Information Center. Likewise, a website must be constructed to feature the project descriptions and team members of the cooperative programs, so that any one with an interest in a particular project/issue can easily review research on that topic. In addition, annual reports on each research program shall be provided at the website and in paper form. Customers can easily see what has been accomplished and comment on how the program can be improved.

PROSPECTS

Meeting Emerging Challenges of Biotechnology

Voluminous reports and information are available addressing issues and concerns on biotechnology. Agricultural biotechnology will undoubtedly play a major role in the creation of products to meet the emerging demand in agriculture. People around the world have developed and are using many kinds of biotechnology. Traditional biotechnology applications have been around for millennia, including manipulating microorganisms in the fermentation process to make bread, beer, wine, soy sauce, cheese and many other food products. It is not until the emergence of molecular biology, that the rapid development of modern biotechnology reached a ground-breaking advancement because of its enormous power to transform living things. New techniques such as tissue culture, cell culture, embryo transfer, animal cloning, as well as various techniques of molecular manipulations and genetic transformation, have emerged and have been widely used. Most prominently, transgenic technologies such as genetically modified organisms/products have been widely used in crop breeding and commercial production.

We can not stop and turn back. Biotechnology is here to stay. For example, the global area planted to genetically modified (GM) crops has been estimated to exceed 80 million ha this year. The rapid expansion of GM crops all over the world has raised public concern. Producers tend to be optimistic about the potential benefits,

and advocate the rapid development and mass production of such crops. Others, however, particularly those who consume the GM products, are concerned about the long-term uncertainty with respect to food safety and the environment. Public acceptance of agro-biotechnology will become universal when products are developed with primary consideration on food safety and quality, health improvement property, and environment-friendly production, among others. More science-based information is needed to unravel such a myriad of concerns and expectation generally brought about by consumers and from the market demand.

I would like to take Taiwan's experience in empowering small-scale farmers for knowledge-based agriculture, as an example, and bring some of my perspectives for your comments, as well as to promote more awareness of the potential benefits of agro-biotechnology research for consideration in our future cooperative program. In reality, as the global economy becomes knowledge-based, the utilization of information and technology will increasingly drive agricultural development. In recognition of the importance of the development of agro-biotechnology, Taiwan has made it a priority to maximize the practical applications of improved technologies at the farm level in the past. Now, the government has likewise expanded efforts to promote commercialization of biotechnology products. Information about basic research in agro-biotechnology needed to be brought to the public domain. Thus, the government now aggressively undertakes research and development initiatives related to biotechnology. Research findings with potential for further application are transferred to the private sector and farmers through various extension channels, either through government agencies or farmers' associations. So far, research findings on agro-biotechnology developed by the public sectors, particularly from the agricultural research institutes, have been applied to production in many ways. These include tissue culture for the production of healthy seedlings, breeding improvements, bioagents (biofertilizer, biopesticide), vaccines, and food processing, among others. Furthermore, to gear up for both research and commercial development, five biotech science parks have been set up by the public sector in different locations around the island. The biotech science parks offer incentives for private companies to join in future efforts to produce and market agricultural biotechnology products. Taiwan's experience in the development of agro-biotechnology, to a greater extent, could benefit not only small-scale farmers and medium enterprises (SME) in the island, but through regional cooperation, the small-hold farmers in the Asian and Pacific region as well. In addition, it is highly desirable that projects listed in the cooperative program should develop a good complementary linkage with the NARS (National Agricultural Research System), but duplications with the NARS' ongoing projects must be avoided.

Like the member countries in the Asian-Pacific Economic Cooperation (APEC), Taiwan is especially interested in the potential of agro-biotechnology to reduce costs and increase productivity and profitability. For example, APEC Ministers specifically addressed biotechnology at its 9th APEC Ministerial Meeting held in Vancouver, Canada in 1997. They asserted that biotechnology "offers the promise of greater food security, safer environments, and enhanced economic development." The APEC joint statement included the following:

> "Recognizing the vital contribution that biotechnology can make toward expanding agricultural and food production, Ministers encouraged the Agricultural Technical Cooperation Experts Group to intensify science-based approaches to the introduction and use of biotechnology products."

Furthermore, in 1999, APEC Leaders in Auckland recognized the important contribution that biotechnology can make in expanding agricultural production, and requested recommendations for future work. Until now, the ATCWG (Agricultural Technical Cooperation Working Group) has listed "Research, Development and Extension of Agricultural Biotechnology" as one of its thrust areas for its work/action plans. All these emphasize our interest and close involvement in the promotion of biotechnology for use in agriculture. I still can recall vividly, as Lead Shepherd of the APEC/ATCWG, the diverse views concerning scientific information on biotechnology. But finally, at the end of the meeting, a consensus was reached to list biotechnology related issues as part of the ATCWG work plans for regional cooperation. Since the USA and Taiwan are members of APEC, our bilateral cooperation in agricultural sciences, which listed biotechnology as one of its priority areas, has had a regional implication and significance.

Strengthen Transgenic Research for Greater Application in Biotechnology

In the midst of trade liberalization and globalization, simply increasing productivity is not necessarily the answer to meet new challenges for agricultural development. High value growth in agriculture by adopting new technologies will be derived from diversification into production of new value varieties/products for emerging market demands. In recognizing this trend, the 21st century will be the century of biotechnology. Biotechnology can play a major role in promoting the growing value of agriculture and in meeting new demands.

You are all more knowledgeable on the potential of biotechnology. So, just allow me to illustrate to you a remarkable case about how Taiwan's scientists have made a breakthrough in animal cloning technology. A team of scientists from the Livestock Research Institute, the National Taiwan University, the National Chung-Hsing University, and the National Pingtung University of Science and Technology have successfully cloned a dairy goat, named Paoyu in March 2004, making her Taiwan's first transgenic cloned goat that carried a foreign gene – a human coagulating blood Factor 8 – in its genome. She has since grown into a healthy goat. Remarkably, the cloned mother Paoyu was impregnated successfully through natural methods in November 2004 and gave birth to a kid in late April 2005. The kid, named Paopei, which carried the same foreign gene deliberately inserted into her mother, has now grown healthy. Both the cloned mother goat Paoyu and the kid Paopei marked a new milestone in Taiwan's animal cloning history. The scientists are jubilant at the birth of Paopei, now growing normally, because it proves that a transgenic cloned goat can reproduce naturally. The

breakthrough in animal cloning technology has given Taiwan headway in opening up new opportunities and challenges in the application of biotechnology.

Enhanced Research Approaches in Potential New Frontiers to Meet Emerging Demands

1. Streamline project management and timely review of cooperative program

I am glad to note that, after reviewing the reports and minutes of the meetings of the cooperative programs, the annual implementation of the projects was very much consistent with the agreed upon priorities of the collaboration program. I noted that the research topics I proposed and which were agreed upon in 2000, which was my final year of involvement in the cooperative program, had been incorporated in the collaboration program, such as the studies on health-related factors like antioxidants in vegetables and fruits, and control of notorious quarantine insect pest like fruit flies. I am likewise pleased to see and would like to applaud the efforts made by both sides for their timely response to the needs of the changing times, and for identifying priority areas not only for current implementation but for redefining directions for future cooperation. I believe that after this workshop, the cooperative program will even be better equipped to refine its vision to look into more challenge areas of research and development for future cooperation in agricultural sciences, particularly in the sphere of agro-biotechnology toward biosafety and quality.

2. Tapping new areas to meet emerging demands

The most recent bilateral meeting between TECRO and AIT on cooperative program in the agricultural sciences was held on 19 April 2005. As in the past, each year, more new specific priority areas are identified to further redefine cooperative research efforts in subject areas described in the Amendment in the Guidelines for the Cooperative Program in Agricultural Sciences between AIT and TECRO. In this year's discussion as recorded in the minutes, the consensus for both sides is to cooperate on the following 2006 priority areas for 2006:

- Genetic engineering of plants and animals and related assessment, management, communication, detection and standards accession;
- Application of bio-chips in agriculture;
- Micronutrients;
- GM regulation;
- Introduction of preventative and diagnostic technique on zoonosis; and
- Plant functional genomics research.

3. Additional areas for future cooperation

To my view, with respect to future priorities for cooperation beyond 2006, research efforts on such areas as genetic transformation of rice crop for enhancing dietary fiber, bio-farming, bio-material research, and soil-rhizosphere system must be pursued.

Genetic Transformation of Rice Crop to Enhance Dietary Fiber

It may be a bit of a wild thinking but I imagine it would be highly beneficial to people with rice as their staple food – to be eating rice which is high in dietary fiber. Similarly, an excellent example of increased nutritional content in foods was nicely exemplified by the breeding success of "golden rice" – the introduction of genes for the production of beta carotene into rice. Although rice is one of the most common crops grown worldwide, it lacks Vitamin A. Consumption of the new variety of rice could help millions of people fight various aspects of Vitamin A deficiency. Importantly, rice is the major staple for many areas of the world. From health aspects, the transformation of rice variety to contain dietary fiber will undoubtedly increase its value for human consumption. Research indicates that a diet high in fat and sugar and low in dietary fiber is damaging to the health, and can result to many diet-related illnesses.

Innovative Approach to Biofarming/ biopharming

Research in biofarming or more specifically biopharming has been addressing widely the potential implications of agro-biotechnology. This type of molecular farming technology involves modifying plants and animals from transgenic genes to produce specific molecules that can be extracted via processing and refinement after production to produce therapeutic proteins, vaccines, and industrial enzymes. This may be a small tonnage market, but the value per unit of weight can be very high. More researches are needed to tap the potential of biofarming/biopharming.

Modern Approaches to Bio-material Research

Bio-material research is another emerging area in the sphere of biotechnology. This refers to harnessing naturally occurring compounds, enzymes, and microbes by molecular tools to enable the production of renewable materials and fuels, and keep the environment clean through biodegradable approach. In addition, many researchers advocate that bio-materials produced from crops will form the foundation for the next generation of hydrogen-based fuel cells for transportation and power generation, and a range of applications not yet developed.

Unraveling the Myth of Soil-rhizosphere System for Efficient Crop Production and Fertilizer Use

Good soil-rhizosphere system is the basic condition to ensure good crop production and quality. To maintain good growth condition, more studies need to be conducted to monitor and identify optimal conditions for soil- rhizosphere system, and cost-effective fertilizer use.

CONCLUSION

In the past two decades, 99 cooperative projects have been conducted by scientists and researchers from both sides, which have yielded many valuable results in terms of both basic and applied studies in agriculture, particularly in the context of agricultural biotechnology. With respect to future priorities for cooperation, both the TECRO and AIT sides have reached a consensus during their bilateral meeting in Taipei, to cover genetic engineering of plants and animals and its related risk assessment, management, communication, detection and standards accession; application of bio-chips in agriculture; micronutrients; GM regulation; introduction of preventative and diagnostic techniques on zoonosis, and plant functional genomics research. This list does not exclude cooperation in other areas such as those identified in the Guidelines for the Cooperative Program. This reiterates the importance that biotechnology has been employed and will play a major role in the science-based improvement of agriculture.

The application of biotechnology to agriculture creates great challenges as well as opportunities. Agriculture is the foundation of many societies in the Asian and Pacific region, and Taiwan is no exception. It serves, not only as a provider of food and livelihood, but also as a very important source of social and economic stability, and a dynamic interaction between human beings and ecosystem. However, like Taiwan, the majority of farmers in the Asian region are engaged in small-scale farming. Their access to new information and technology is relatively poor. Production is labor-intensive. It has been my thinking for some time – that it is possible that the significant results of many of the past US-Taiwan bilateral collaborative research projects could be built into a mechanism of transferring technology and knowledge gained through regional cooperation. Another possibility is to utilize the long-standing Taiwan Agricultural Technical Mission as a stepping stone to transfer technology and assist the agriculture sectors of developing countries. The TECRO-AIT agricultural science cooperation projects pose much benefit of regional implications. In addition, given the current trends in globalization and trade liberalization, the small-hold farmers need to be able to take advantage of new technical developments, since they are competing with big farmers all over the world. Through such regional cooperative efforts, information about appropriate biotechnology may be extended and transferred as needed to countries primarily dependent on small-scale farming.

It is a delightful opportunity for me to participate in this workshop, and to join you in celebrating the 20th year anniversary of the TECRO-AIT Cooperative Programs in Agricultural Science. The locomotive of biotechnology train is running fast and I anticipate seeing you in the next workshop catching up on this trend in not too far future.

A Scientist's View of Experiences Gained from the USDA FAS-Taiwan COA Joint Research Program: Accomplishments and Impact on Management of Viral Diseases of Ornamental Plants

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ABSTRACT

In 1989, a three-year collaboration project on "Rapid methods for diagnosing ornamental plant viruses and the control measures" among National Chung-Hsing University (NCHU), Taiwan Agricultural Research Institute (TARI) and the Floral and Nursery Plants Research Unit (FNPRU, formerly Floral and Nursery Crops Laboratory) of the U.S. National Arboretum of the United States Department of Agriculture (USDA) was initiated through the USDA Foreign Agriculture Service (FAS)-Taiwan Council of Agriculture (COA) Cooperative Research Program. Subsequently, three additional projects were awarded to Feng-Shan Tropical Horticultural Research Institute, NCHU, TARI, Taichung District Agricultural Improvement Station and Taiwan Seed Propagation Station allowing their investigators to collaborate with scientists at FNPRU. The joint studies are highly productive and have published in various international journals. A novel method for the detection of viruses and phytoplasma in infected plants was developed. Several previously undescribed new viruses infecting ornamental plants were discovered and characterized. A method for efficient production of antigens for immunization was developed and specific rabbit antisera and mouse monoclonal antibodies were produced. Results from USDA FAS-Taiwan COA joint studies have enormous impact on sound management of viral diseases in floral crops as well as the advancement of plant virology.

Key words: Scientist's view, USDA FAS-Taiwan COA Joint Research Program, Management, Viral diseases, Ornamental plant.

INTRODUCTION

For many years through the United States Agency for International Development (USAID), United States-Israel Binational

Agriculture Research and Development Fund (BARD), and Special Foreign Currency Program (PL 480 projects), the Floral and Nursery Plants Research Unit (FNPRU) of the U.S. National Arboretum of the United States Department of Agriculture (USDA) has been working closely with overseas scientists solving plant health problems related to viral diseases. With Taiwan scientists, the collaborations are carried out through the USDA Foreign Agriculture Service (FAS)-Taiwan Council of Agriculture (COA) Cooperative Research Program. The program started in 1968, and in 1989 a three-year collaboration project on "Rapid methods for diagnosing ornamental plant viruses and the control measures" among scientists at National Chung-Hsing University (NCHU), Taiwan Agricultural Research Institute (TARI) and FNPRU (formerly Floral and Nursery Crops Laboratory) was implemented. Subsequently, three additional projects were awarded to Feng-Shan Tropical Horticultural Research Institute, NCHU, TARI, Taichung Agricultural Improvement Station and Taiwan Seed Propagation Station allowing their investigators to collaborate with scientists at FNPRU. The collaborations are highly productive, and our studies have resulted in numerous joint publications in various international journals and reviews. This communication highlights achievements made by investigators on research funded through the USDA-COA Collaborative Research Program.

Accomplishments

1. Calla lily chlorotic spot virus

A new tospovirus, Calla lily chlorotic spot virus (CCSV), was isolated from calla lilies (*Zantedeschia* spp.) (3,9). It causes chlorotic spots, ranging from light green to yellow, on the middle leaves of the affected plants. Virions are about 75 to 105 nm, similar in size to other tospovirus particles reported. CCSV is transmitted by *Thrips palmi*. The virus is serologically but weakly related to *Watermelon silver mottle virus* (WSMoV). Results of reverse transcription-polymerase chain reaction (RT-PCR) show that the conserved regions of the L genes of tospoviruses are present in CCSV. The CCSV S RNA is 3,172 nucleotides (nt) in length, with an inverted repeat at the 5′ and 3′ ends and two open reading frames (ORFs) encoding the nucleocapsid protein (NP) and a nonstructural (NSs) protein in an ambisense arrangement. A typical 3′-terminal sequence (5′-AUUGCUCU-3′) that is shared by all members of the genus *Tospovirus* also is present in the CCSV S RNA. The CCSV NP and NSs protein share low amino acid identities, 20.1 to 65.1% and 19.9 to 66.1%, respectively, with those of reported tospoviruses. Phylogenetic dendrogram analysis indicates that CCSV is a distinct member in the genus *Tospovirus*. The results indicate that CCSV is a new species within the WSMoV serogroup in the genus *Tospovirus*.

2. Tospovirus nucleocapsid proteins

An *in vivo* infectious clone of *Zucchini yellow mosaic virus* (ZYMV) was engineered to express the NPs of tospoviruses *in planta* (5). The open reading frames of NPs of different serogroups of tospoviruses, including *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV), *Watermelon silver mottle virus* (WSMoV), *Peanut bud necrosis virus* (PBNV), and *Watermelon bud necrosis virus* (WBNV), were in frame inserted in frame between the P1 and HC-Pro genes of the ZYMV vector. Six histidine residues and an NIa protease cleavage site were added at the C-terminal region of the inserts to facilitate purification of expressed NPs. Approximately 1.2-2.5 mg NPs per 100 g tissues were purified from leaf extracts of inoculated zucchini squash. Using the expressed WBNV NP, rabbit polyclonal antisera and mouse monoclonal antibodies were produced. The procedure provides a rapid and convenient method for production of large quantities of pure tospoviral NPs *in planta*. The system also has a potential application for the production of other proteins of interest in cucurbits.

3. Calla lily latent virus

A new potyvirus, *Calla lily latent virus* (CLLV) was isolated from apparently healthy calla lilies (*Zantedeschia* spp.) and characterized (2). CLLV infects *Chenopodium quinoa* and develops local lesions on inoculated leaves. Virions are approximately 780 nm long. CLLV is transmitted readily to and established in *C. quinoa*. The virus is, however, transmitted from infected to healthy calla lilies with difficulty. A 1.3-kb cDNA is produced by RT-PCR from CLLV-infected calla lilies and *C. quinoa* using potyvirus degenerate primers. The PCR product was cloned and sequenced. It consists of 1,339 nucleotides (GenBank Accession No. AF469171) corresponding to the genome organization of the 3′ terminal region of potyviruses. The deduced amino acid sequence contains 362 residues comprising the 3′ terminal region of the nuclear inclusion b protein (80 residues) and the complete coat protein (CP) (282 residues). A 253 nt-noncoding region (NCR) is located at the 3′ terminal region of the cDNA. Establishment of CLLV as a new species of *Potyvirus* is based on the uniqueness in the CP gene and 3′ NCR. Polyclonal antibodies produced to *E. coli*-expressed CLLV CP are useful for the detection of CLLV and its CP in calla lilies in enzyme-linked immunosorbent assay (ELISA), sodium dodecyl sulfate (SDS)-immunodiffusion assay, immuno-specific electron microscopy, and western blot. In field surveys, calla lily plants found positive for CLLV by serological methods always remain symptomless throughout the six-month growing season. Occasionally, CLLV is detected in symptomatic calla lilies dually infected with other viruses (*Dasheen mosaic virus* or *Konjak mosaic virus*).

4. New potyviruses associated with golden spider lilies

Two potyviruses associated with golden spider lily severe mosaic disease were identified (1,4). Inoculum prepared from severe mosaic tissue was able to induce local lesions on *Chenopodium quinoa* whereas inoculum prepared from mild mottle tissue failed to infect *C. quinoa*. Tests with 25 known potyvirus antisera failed to detect the presence of viruses in infected spider lilies by ELISA. The antigens, however, reacted with potyvirus-specific monoclonal antibodies. Electron microscopy revealed the presence of flexuous rod-shaped virus particles and pinwheel inclusions in leaf dips and ultra-thin sections of infected severe mosaic tissues, respectively. Purification resulted in typical potyvirus particles measuring about 750 nm. Two species of protein with estimated relative mass of 35 and 37 kDa were detected from samples purified from tissues bearing severe mosaic symptoms. An antiserum was subsequently produced. Following the same purification procedure, only a 37-kDa protein species was detected in the sample purified from mild mottle tissue. In SDS-immunodiffusion

test, antiserum reacted with its homologous antigen prepared from severe mosaic tissues and produced two precipitation bands. It also reacted with the antigen extracted from mild mottle tissue by producing only one single precipitin line. The results indicate that the severe mosaic disease of golden spider lily is caused by two serologically distinct potyviruses and that one, provisionally named *Lycoris mild mottle virus*, induces mild mottle symptoms. The other potyvirus, capable of inducing local lesions on *C. quinoa*, is tentatively named Lycoris potyvirus.

5. Tissue blot immunoassay

A technique involving blotting of tissue on nitrocellulose membranes was described for the detection of plant viruses and a phytoplasma in infected plants (6-8). Tissue blots are prepared by pressing, with a firm and gentle force, the freshly cut tissue surface on nitrocellulose membranes. Antigens in tissue blots on nitrocellulose membranes are detected by enzyme-labeled immunological probes. In indirect immunological methods, the blots are reacted with antigen-specific primary antibodies and detected with enzyme-labeled species-specific secondary antibodies. Alternatively, the blots are reacted with antigen-specific biotintylated primary antibodies and detected with avidin-enzyme conjugates. In direct immunological methods, the blots are reacted and detected with enzyme-labeled antigen-specific antibodies. The unique and novel procedure of tissue blot immunoassay has become one of the standard methods for the investigation of plant pathogens. It enables diagnostic technicians to handle large numbers of test samples. This method has been adopted by an ARS Research Plant Pathologist and applied to virus indexing. It is now utilized in citrus tristeza virus certification in citrus orchards by a private diagnostic laboratory (Nokomis Corp., Altamonte Springs, FL 32715, USA).

6. Engineering Cymbidium mosaic virus resistance in orchids

Full-length heavy and a light chain cDNAs (1545 and 948 bp), CymMV-H 10-1 and CymMV-L 23, respectively, were selected by hybridization with specific oligonucleotide probes (10). The sequence of the CymMV-H 10-1 cDNA clone (GenBank Accession Number AY571287) contains a 5′ untranslated region (37 bp, nt 1-37), an open reading frame (1386 bp, nt 38-nt 1423) including a 19 amino acid leader peptide sequence (57 bp, nt 38-94), a variable region (339 bp, nt 95-433) and a constant region (990 bp, nt 434-1423). The sequence of the 3′ end of this clone contains an untranslated region (102 bp, nt 1424-1525) with a prototype sequence of AATAAA (nt 1492-1497) at a position 28 nucleotides upsteam of the poly A region (nt 1526-1545). The sequence of the CymMV 23 cDNA clone (GenBank Accession Number AY571284) contains part of the 5′ untranslated region (2 bp, nt 1-2), an open reading frame (720 bp, nt 3-722) including a 20 amino acid leader peptide sequence (60 bp, nt 3-62), a variable region 336 bp, nt 63-398) and a constant region (324 bp, nt 399-722). The sequence of the 3′ end of this clone contains an untranslated region (208 bp, nt 723-930) with a prototype sequence of AATAAA (nt 908-913) at a position 17 nucleotides upstream of the poly A region (nt 931-948). The results in this study are a step closer in the exploration of transgenic plants expressing a functional single-chain variable fragment antibody specific to the viral coat proteins for control of CymMV. In addition, a recombinant pBin19 vector containing CymMV coat protein cDNA was transformed into Dendrobium by particle bombardment. Results of transformants are now being evaluated.

CONCLUSION

Early detection and accurate identification of causal agents are among essential strategies for a successful management of diseases caused by viruses. The identity of a specific virus determines the control measures to be adopted, such as the removal and eradication of the source of infection and the control of vector populations in the field and its proximity. Detection of virus infection is usually accomplished by means of serological and/or nucleic acid-based techniques. Results from USDA FAS-Taiwan COA joint studies have enormous impact on sound management of viral diseases in floral crops as well as the advancement of plant virology. Studies led by Shyi-dong Yeh at the Department of Plant Pathology of NCHU are at the forefront in the area of tospovirus research. His laboratory is one of the two leading centers in the world conducting research on tospoviruses. He is a member of the International Working Group of Tospovirus since 1995 when an International Symposium of Tospoviruses and Thrips of Floral and Horticulture Crops was held in Taiwan. Chin-an Chang of Plant Pathology Division at TARI is a vanguard in the research area of ornamental diseases caused by viruses. He has gained recognition among his colleagues and is a member of the Council of International Working Group on Ornamental Viruses since 2000. In 2004, he organized and convened the 11th International Symposium on Virus Diseases of Ornamental Plants in Taiwan. All collaborating investigators actively participated in and presented papers at both symposia. The opportunity of collaboration through USDA FAS-Taiwan COA Cooperative Research Program that increases contact with other scientists throughout the world working in the academic community is a great reward to each scientist involved.

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Studies on Viruses Infecting the Family of *Amaryllidaceae* and Development of Their Detection Techniques

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ABSTRACT

The family of *Amaryllidaceae* contains abundant plant species adaptive to diversified environment and numerous of them are artificially grown as ornamental plants. Taiwan is the native country of many amaryllis plant species such as the well known golden spider lily (*Lycoris aurea* Herb.). Many species in amaryllis family are considered to have great potential transforming into novel ornamental crops in the future. To pursue this goal, we believe detailed understanding of virus diseases that may threaten the growth and production of amaryllis plants is crucial. Most amaryllis plants and many other ornamentals are propagated solely by vegetative means so that their seedlings will be jeopardized once mother stock planting materials are infected by viruses. During last four years, virologists in Taiwan Agricultural Research Institute have been in close cooperation with scientists from USDA/ARS, Beltsville Agricultual Research Center, USA to survey the viruses infecting various species of the family *Amaryllidaceae*. At present, we have succeeded in identification and characterization of six viruses infecting amaryllis plant species including *Lycoris*, *Narcissus*, *Hippeastrum* and *Agapanthus*. At least four of these six viruses are new records in the literature and named by this research team. Specific antibodies and nucleic acid primers and probes against these viruses were successfully produced and their standard detection procedures were established. These detection kits and protocols are essential and vital to the development of virus-free amaryllis plant propagation system. It is hoped that with this accomplishment the future development of amaryllis ornamental industry in Taiwan and USA will both be benefited.

Key words: *Amaryllidaceae*, Viruses, Detection, Characterization, *Lycoris*, *Narcissus*, *Hippeastrum*, *Agapanthus*.

INTRODUCTION

There are 90 genus and probably over 1200 plant species recorded in the family of Amaryllidaceae [(8)]. Among them several Amaryllis genus such as *Narcissus*, *Hippeastrum*, and *Nerine* are well known world widely for their ornamental importance [(8)]. In recent years, another genus of this family, *Lycoris*, has absorbed attention from botanists, breeders and horticulturists around the world for its

diversities in the color and morphology of flower parts and especially its broad adaptation abilities to different environmental conditions [8]. It is proposed that *Lycoris* shall be one of the most promising new ornamental crops in the coming twenty-first century. Since Taiwan is the origins of several *Lycoris* ornamental plant species, we certainly are advantaged in the development of this ornamental crop. However, in the history of ornamental plant industry development, the grasp of disease control techniques especially those for viral agents are known to be one of the keys to success. The experience of Dutch ornamental plant industry and their leading technology in virus indexing program certainly have explained very well this situation. Therefore, to ensure the strong competition in the future's *lycoris* industry, we proposed that the research program on the virus diseases of *lycoris* or even in the whole family of Amaryllidaceae should be established as soon as possible. Since 2001, we started a three-year collaborative project on "Studies on viruses infecting the family of Amaryllidaceae and development of their detection techniques" with the Floral and Nursery Plants Research Unit (FNPRU, formerly Floral and Nursery Crops Laboratory) of the U.S. National Arboretum of the United States Department of Agriculture (USDA). Presently, we have succeeded in identification and characterization of six viruses infecting amaryllis plant species including *Lycoris*, *Narcissus*, *Hippeastrum* and *Agapanthus* (Table 1)[1, 2, 3, 4, 5]. Four of these six viruses are new records in the literature and named by this research team. Specific antibodies and nucleic acid primers and probes against these viruses were successfully produced and their standard detection procedures were established. We are confident that with this accomplishment the future development of amaryllis ornamental industry in Taiwan and USA will both be benefited.

ACCOMPLISHMENT

1. Lycoris viruses

Golden spider lily (*Lycoris aurea* Herb.) is an indigenous species and currently a popular cut flower ornamental bulb crop in Taiwan [8]. There have been twenty-four different viruses documented in the literature infecting plants of *Amaryllidaceae* [2]. Most of these 24 viruses are from the reports of three currently most well-known genera in the *Amaryllidaceae*, i.e. *Narcissus*, *Hippeastrum*, and *Nerine*. For *Lycoris*,

Table 1. Viruses found in Taiwan that infect species of *Amaryllidaceae*.

Virus	Genus	Host
Lycoris mild mottle virus (LyMMV)	*Potyvirus*	Golden spider lily (*Lycoris*)
Lycoris potyvirus (LPV)	*Potyvirus*	Lycoris, Narcissus
Lycoris virus T (LPV)	*Carlavirus*	Lycoris, Narcissus, Hippeastrum
Hippeastrum mosaic virus, isolate HIP4	*Potyvirus*	Hippeastrum
Narcissus virus isolate WN9 could be a strain of *Narcissus yellow stripe virus*	*Potyvirus*	Narcissus
African lily mosaic virus	*Potexvirus*	African lily (*Agapanthus*)

however, there hasn't any viruses been reported until recently our laboratory documented that at least two new potyviruses and a unreported carlavirus were found associating with severe mosaic disease of golden spider lilies (*Lycoris aurea*) [1, 2, 5]. The 3'-terminal region of the genome of these two potyviruses were separately amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using a potyvirus-specific degenerate primer set. The amplicons were cloned and subsequently sequenced. They were found corresponding to the genome organization of potyviruses with a coat protein (CP) gene followed by a 3'-noncoding region. Comparing these sequences with each other and with those reported in the GenBank, these two lycoris potyviruses were revealed to be different from each other and also unique to other known *Potyvirus* species [2]. One of the lycoris potyvirus was named as *Lycoris mild mottle virus* (LyMMV) because it was frequently found singly infected some golden spider lily plants developing mild mottling symptom on their leaves [2]. The second potyvirus was provisionally designated as *Lycoris potyvirus* (LPV) for its specific symptom on golden spider lily was not yet conclusive [2]. PCR primers for specific amplification of LyMMV and LPV were designed based on their 3-terminal genome sequences and the primers were successfully used for the detection and identification of these two potyviruses in golden spider lilies. Specific antibodies against LyMMV and LPV were separately prepared in rabbits by immunizing viral coat protein expressed in *E. coli* using expression vector plasmid pET28b(+) (Novogen, Inc., Madison, WI, USA). The antibodies were shown feasible to differentiate these two lycoris potyviruses from each other and also from other known potyviruses. They could also be applied in ELISA, SDS-immunodiffusion and Western blotting tests for the virus indexing in plants.

In subsequent studies, another virus isolate was obtained from lycoris plants exhibiting strong mosaic symptoms. Different from LyMMV and LPV, the isolate was able to induce local lesions on *Chenopodium quinoa*, which was therefore routinely used to maintain pure culture of this new virus isolate. After characterization studies, this new isolate was found to be a carlavirus [5]. Its virus particles are flexuous rod shaped, about 617 nm in length, typical of carlaviruses. A sequence of 8047 nucleotide representing almost entire genome, except probably less than a hundred nucleotides from the 5'-terminal non-coding region, of this isolate was sequenced. The genome organization was found corresponding to carlaviruses with a typical triple gene block in the 3' part. A coat protein gene containing 894 nucleotides which encodes 298 amino acid residues was revealed. Comparing with other known carlaviruses in the GenBank, the percent identities of the nucleotide and amino acid sequence of CP gene were less than 60%, showing that the isolate obtaining from lycoris should be recognized as a new carlavirus species [5]. We proposed that the virus is named as *Lycoris virus* T (LVT). A polyclonal antiserum against LVT was successfully prepared in rabbits by immunizing virus particles purified from infected tissues of *C. quinoa*. The antiserum was found to be useful to detect LVT infection in ELISA and many other serological tests. Specific PCR primers for the detection and identification of LVT was

also designed and routinely applied in regular virus surveys task.

2. *Narcissus viruses*

Narcissus is an important and popular ornamental bulbous crop worldwide. Two species of narcissus, namely the Chinese narcissus (*Narcissus tazetta* L. var. orinentalis Hort.) and the western narcissus (*N. poeticus*) are the major commercial ones in Taiwan. Virus symptoms of mosaic, mottling and yellow striping on the leaves are commonly seen on narcissus plants for sale in the markets. Using potyvirus-specific degenerate primers, two molecularly different potyvirus isolates, namely CN4-2 and WN9, were frequently found associating with Chinese narcissus plants bearing viral symptoms [9]. However, in western narcissus only WN9-related virus isolates were detected [9]. Based on our observation, WN9-infected narcissus plants always exhibited yellow striping and mild mosaic symptoms on their leaves, which was similar to those described for *Narcissus yellow stripe virus* (NYSV) in the literature. However, due to the lack of documentation of NYSV's genome sequence in GenBank, we are not sure presently whether our WN9 isolates are strains of NYSV or a unique potyvirus species since its CP gene sequence shares less than 80% identities with other known potyviruses.

CN4-2 from Chinese narcissus was later determined to be molecularly similar to LPV found in golden spider lily [9]. Comparative studies showed that they shared more than 95% of identities in nucleotide and amino acid sequences of CP gene, indicating they are strains of the same virus species. An antiserum prepared against *E. coli* expressed coat protein of CN4-2 was found reacting strongly in ELISA with isolates of LPV from lycoris [3]. Using this antiserum, LPV isolates from lycoris and CN4-2 from narcissus were found serologically indistinguishable in SDS- immunodiffusion test [3]. We have also prepared antiserum against bacteria expressed CP of WN9 and shown that it could be applied in ELISA and many other serological tests to detect and identify virus infection in narcissus and other ornamental plants [3].

African lily

African lily (*Agapanthus africanus* L.), a species of *Amaryllidaceae* and native from South Africa, has become a popular ornamental crop in Taiwan and many other countries. It can be used as bedding plants or intensively grown for cut flower production. In 2002, we observed a diseased African lily plant with mosaic symptom on its leaves in Nantou County [4]. The plant was brought back to the lab and inoculated on various indicator plants. Local lesions were found on *Chenopodium quinoa* 7-10 days after mechanical inoculation. Single lesions were taken and re-inoculated on the same plant and this procedure was repeated for three times to obtain pure culture of this virus isolate which was named as AL-1 [4]. Serologically, none of the antisera against more than 30 different known viruses in our collection reacted with AL-1 [4]. In electron microscopy, flexuous rod shaped virus particles with 508 nm in length were consistently observed in negatively stained leaf dips and samples purified from *C. quinoa* [4]. Analyses of purified virus sample in SDS-PAGE showed that the virus contained a single species of protein subunit with a molecular mass about 23 kDa [4]. An antiserum prepared against purified virion of AL-1 reacted strongly with its homologous antigen from *C. quinoa* and also

with diseased African lily plants, but not with non-symptomatic African lilies and non-inoculated *C. quinoa* in SDS-immunodiffusion and ELISA tests. Interestingly, a potexvirus-specific degenerate primer set was shown to react with AL-1 and to amplify a 280 bp product equivalent to the size of amplicons amplifying from potexviruses [4]. Subsequent molecular studies have cloned and sequenced a 2.6 kb fragment corresponding to the 3'-terminal region of AL-1's genome, which was also found coinciding with that of potexviruses [4]. Triple gene blocks containing a CP gene were detected in this 2.6 kb sequence. CP gene of AL-1 was found to contain 660 nucleotides with 220 deduced amino acid residues. Comparing with 18 CP gene sequences of known potexviruses in GenBank, AL-1 shared only 54% and 38% identities in nucleotide and amino acid sequences, respectively [4]. This result indicates AL-1 isolated from African lilies is a newly recognized species in the Genus of *Potexvirus* and it was named as *African lily mosaic virus* [4].

Amaryllis

Amaryllis is commonly grown in Taiwan as potting or bedding ornamentals. We obtained a virus isolate (HIP) from a diseased amaryllis plant showing systemic mosaic symptoms, which is commonly seen in amaryllis nurseries [6,7]. A 2-kb DNA product was amplified by RT-PCR from HIP by the use of potyvirus-specific degenerate primers [6,7]. The PCR product was subsequently cloned and its sequence analyzed. It was found comprising of 1851 nucleotides (nts) corresponding to the 3'-terminal region of potyviral genome. The deduced amino acid sequence contained 576 residues encoding part of the 3'-terminal region of NIb gene (264 residues) and the complete sequence of coat protein (CP) gene (312 residues) [6, 7]. A 122 nts of non-coding region (NCR) was found locating at the 3'-terminal region of the DNA. Comparing HIP CP gene with those of known potyviruses in GenBank, the percent identities of nucleotide and amino acid sequence were less than 53% and 45%, respectively [6, 7]. Since genome sequence of *Hippeastrum mosaic virus* (HiMV), the only potyvirus reported to infect amaryllis, has not been reported yet, the identities of HIP and its relationship with HiMV remain to be further explored. Based on HIP sequence, we developed a specific primer set for RT-PCR detection of HIP and confirmed that HIP is consistently associated with amaryllis plants showing mosaic symptoms [6,7]. Using directional cloning techniques, a 37-kDa fusion protein containing complete CP sequence of HIP and partial sequence encoded by the expression vector plasmid (pET-28b, Novagene), was highly expressed and purified from *E. coli* cell cultures [6, 7]. Antiserum prepared against this fusion protein was applicable in ELISA and SDS-immunodiffussion test for the detection of HIP in amaryllis plants. It did not react with antigens of healthy amaryllis plant and at least 20 different potyviruses in our collections [6, 7].

In a recent survey, some amaryllis plants were found positively reacted in ELISA with antiserum against LVT, a carlavirus found in Taiwan infecting golden spider lilies. Diseased plants were tested in RT-PCR by the use of LVT-specific primers and a DNA product equivalent to the expected size was consistently amplified. The DNA product was cloned and

sequenced. The sequence was found almost identical to that of LVT, confirming that a strain or even the same virus was capable of infecting amaryllis plants.

CONCLUSION

It is generally accepted that successful control of plant virus diseases relies on early and accurate identification of the causal viruses. Knowing the identity of causal virus leads to correct understanding of the mode of transmission and type of vector possibly involved in the disease epidemic. Appropriate control strategy can be thus proposed and implemented in time to suppress disease epidemic. For vegetatively propagated crops such as most ornamentals, accurate identifying mother stock growing materials to be free from virus infection is a crucial step before mass production. Therefore, development of accurate and sensitive virus detection protocol is inevitable for almost any ornament crops attempting to set up industrialized production system. Since 2000, we have been in close cooperation with Dr. Hei-Ti Hsu and his associates from USDA/ARS, Beltsville Agricultual Research Center, USA to survey the viruses infecting golden spider lily, narcissus, African lily and amaryllis in the family *Amaryllidaceae*. At present, we have successfully identified and characterized six viruses including four potyviruses, one carlavirus and one potexvirus. At least four of the viruses are identified to be new species and named by this research team. Specific antibodies and nucleic acid primers and probes against these viruses were successfully produced and their standard detection procedures were established. These detection kits and protocols are essential and vital to the establishment of virus-free planting material propagation system. This fruitful results accomplished by the joint efforts from virologists of TARI and USDA-ARS will certainly benefit the future development of amaryllis ornamental plant industry not only in Taiwan and USA but also other countries around the world.

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Molecular Cloning of Chalcone Isomerase Genes from Soybean and Its Application in Color Engineering

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ABSTRACT

Flower color is an important trait of ornamental flowers. Some beautiful flower crops and varieties are lack of specific colors. For this purpose, the collaborative proposal includes pigment gene cloning, transformation vector construction, gene transformation, and mass propagation of transgenic plants in order to develop new flower (or leaf) colors for chrysanthemum, caladium and anthurium. Three full length chalcone isomerase (CHI) cDNAs have been cloned from the black seed coats of soybean among them CHI1 belongs to type II and CHI2 and CHI3 belong to Type I, respectively. These three CHI genes may with different function and role in the synthesis of isoflavanone and anthocyanins. Theoretically, the yellow flower can be achieved by accumulation of yellow narigenin chalcone in the flavonoid biosynthetic pathway by inhibition of endogenous CHI gene in flower through anti-sense or RNAi technology. For this purpose, the CHI cDNAs were constructed into the pCAMBIA series vectors driven by a 35S promoter in either sense or anti-sense for agrobacterium-mediated transformation to ornamental crops such as, chrysanthemum, caladium and anthurium to obtain yellow color which are not commercially available in flower market. The CHI1 gene has been successfully transferred into chrysanthemum in either sense or anti-sense direction. Flower color of transgenic caladium Red Star with sense CHI construct turns from red to green indicating that the overexpressed soybean CHI gene suppresses the endogeneous CHI gene expression and blocked ntocyanin accumulation on leaves. This result provides an alternative way to engineer color through transformation of the CHI gene. As we know, this is the first report on color engineering in ornamental plants by transformation of CHI gene.

Key words: Soybean, Chalcone isomerase, Color, Caladium, Chrysanthemum, Transformation.

INTRODUCTION

There are always desires for diversities of flower colors from the point of view of consumer therefore who can create new varieties in a short time would have strong competent in market (Mol *et al.*, 1995). From all prospects, the genetic transformation will fit this acquirement properly (Tanaka *et al.*, 1998). Flower colors

are decided by many factors and among them pigments of anthocyanin is the major group (Inagaki *et al.*, 1999). Anthocyanin pathway is one of unique and well-studied pathway of plant however most studies were focused in few model plants like maize, snapdragon and petunia (Mol *et al.*, 1989; Oud *et al.*, 1995; van Houwelingen *et al.*, 1998). Among all genes in anthocyanin pathway, DFR (dihydroflavonol reductase) gene has played an important role for converting dark colors like orange, brick red and purple (Tanaka *et al.*, 1998; Mol *et al.*, 1989). It is thought that by transferring proper DFR gene(s) into the plants lack of key enzyme would accomplish in the very end of anthocyanin pathway to make dark colors theoretically. In the past few years in our laboratory we cloned seven groups of pigment genes including: 4CL, CHS, CHI, F3H, F3'H, DFR, F3'5'H, and GST genes from a black seed coat soybean 'Chin-Ren-Woo-Dow' (CRWD).

Biolistic particle bombarment and *Agrobacterium*-mediated transformation are the most popular two methods used for plants and both methods have many successful examples in various species (Kikkert, 1993; Morrish *et al.*, 1993; Hooykaas and Schilperoort, 1992; Sanford *et al.*, 1993; Smith and Hood, 1995) . In this study, the *Agrobacterium*-mediated transformation method was applied to pigment gene transformation for engineering new colors for both flower and ornamental leaf crops such as chrysanthemum and caladium.

MATERIALS AND METHODS

Plant materials

Seed coats of soybean were harvested from developing seeds of CRWD variety with the *iRT* genotype. Total RNA was isolated by BSA-PVPP-PK method (Wang and Vodkin, 1994) for RT-PCR, northern hybridization and construction of cDNA library. Most molecular techniques were followed the protocols of Sambrook *et al.* (1989) if there is not specified.

Construction of a CRWD cDNA library

The poly(A)$^+$ RNA was prepared from the total RNA using a Dynabeads mRNA Purification Kit (Dynal-Product No.610.01). The cDNA was prepared using 5μg of poly(A)$^+$ RNA according to the instructions for a cDNA Synthesis Kit, ZAP-cDNA Synthesis Kit, and ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene). The first-strand cDNA was synthesized using MMLV-RT. The second-strand synthesis, *Escherichia coli* ribonuclease H and *E. coli* DNA polymerase I were used. The cDNA was ligated to *Eco*RI adaptors, and then cloned into the *Eco*RI site of Uni-ZAP XR vector, packaged and plated on selective XL1-Blue MRF' cells. The result library contained ca. 1×10^6 p.f.u. independent clones.

Genomic library construction and screening for CHI gene

The soybean genomic library was constructed using genomic DNA of CRWD (*iRT* genotype) variety (C. S. Wang *et al.* unpublished) for screening *CHI* gene.

Molecular cloning of CHI2 and CHI3 genes by genome walking

Genome walking technique developed by BD Biosciences (GenomeWalker Kit) was applied for cloning the flanking sequences of CHI genes from soybean genome. A primer, 5'-TGCTCGACTCCCTTGACTTCTACAG-3', was designed for the first round of genome walking. Two DNA fragments were amplified from *Dra* I and *Pvu* II libraries

constructed from restriction enzyme digested genomic DNA of UC9 (*iRT* genotype). To amplify the entire *CHI2* gene, a new primer, 5′-TTGCATGCCTTGA AGATCTGATAAC-3′, was designed for second round of genome walking. A specific DNA fragment (3.0 kb) amplified from *Eco*R V library was cloned and sequenced to complete the *CHI2* genomic sequences. A partial 3′ end gene sequences of the third CHI gene, *CHI3*, was found in the opposite direction by BLAST analysis. The amino acid sequences *CHI1* and *CHI2* are around 70% identical to the Type II CHI of leguminous plants and show low identity to non-leguminous plants (Table 1 and 2). Type I CHI only recognizes 6′-hydroxylasechalcone as a substrate, however, type II CHI catalyzes both 6′-hydroxychalcone and 6′-deoxychalcone.

Table 1. The comparison of amino acids sequence identity of soybean CHI1 with the same proteins published in Genbank.

Species	Identity (%)	Accession No.
Legume		
Pueraria lobata	87	BAA09795
Phaseolus vulgaris	82	CAA78763
Medicago sativa	77	AAB41524
Lotus japonicus CHI3	71	BAC54038
Lotus japonicus CHI1	69	BAC53983
Lotus japonicus CHI4	71	CAD69022
Pisum sativum	68	AAA50174
Non-legume		
Dianthus caryophyllus	53	CAA91931
Petunia hybrida CHIB	53	CAA32730
Ipomoea batalas	52	BAB58538
Ipomoea purpurea	53	AAB86474
Elaeagnus umbellate	53	AAC16013
Lotus japonicus CHI2	51	BAC53984
Vitis vinifera	52	CAA53577
Saussurea medusa	51	AAM48130
Citrus sinensis	52	BAA36552
Allium cepa	48	AAU11844
Petunia hybrida CHIA	50	CAA68769
Callistephus chinensis	50	CAA91921
Eustoma grandiflorum	49	BAD34458
Oryza sativa	4	AAO65886
Raphanus sativa	48	AAB87071
Arabidopsis thaliana	48	CAB94968
Zea mays	48	CAA80441
Hordeum vulgare	46	AAM13449

Table 2. The comparison of amino acids sequence identity of soybean CHI2 with the same proteins published in Genbank.

Species	Identity (%)	Accession No
Legume		
Phaseolus vulgaris	68	CAA78763
Pueraria lobata	69	BAA09795
Glycine max CHI1	69	AAK69432
Medicago sativa	66	AAB41524
Lotus japonicus CHI3	67	BAC54038
Lotus japonicus CHI1	67	BAC53983
Lotus japonicus CHI4	68	CAD69022
Non-legume		
Ipomoea batalas	56	BAB85838
Elaeagnus umbellate	57	AAB86474
Ipomoea purpurea	55	AAC16013
Vitis vinifera	53	CAA53577
Saussurea medusa	51	AAM48130
Lotus japonicus CHI2	56	BAC53984
Petunia hybrida CHIB	54	CAA32730
Callistephus chinensis	52	CAA91921
Dianthus caryophyllus	53	CAA91931
Petunia hybrida CHIA	50	CAA68769
Citrus sinensis	55	BAA36552
Allium cepa	47	AAU11844
Eustoma grandiflorum	52	BAD34458
Pisum sativum	57	AAA50174
Arabidopsis thaliana	50	CAB94968
Raphanus sativa	49	AAB87071
Zea mays	51	CAA80441
Hordeum vulgare	46	AAM13449
Oryza sativa	47	AAO65886

Pigment gene construction, transformation and transgenic plant screening

The cDNA sequence of CHI has been inserted into pCambia vector series for agrobacterium-mediated transformation. Both sense and antisense vectors are constructed for transformation purpose. The sense pLMGDFR1GUS and anti-sense pLMGDFR1AGUS plasmid consisting of a *uidA* reporter gene coding for β-glucuronidase (GUS) and a hygromycin phosphotransferase (*hpt*) selectable marker gene was used for transformation. Both genes are controlled by the cauliflower mosaic virus (CaMV) 35S promoter and followed by nopaline synthatase (NOS) polyadenylation sequences. Binary vectors of sense pLMGDFR1GUS and anti-sense pLMGDFR1AGUS plasmid in *Agrobacterium*-mediated transformation system will also be

tested in this project for these two flower crops. Genomic DNA was extracted from each regenerants screened from the antibiotic-containing medium. PCR and Southern hybridization were applied to screen and/or confirmed the transgenic plants. The putative transgenic plants were carefully planted in the greenhouse for flower color investigation and pigment analysis.

Callus induction and shoot regeneration of anthurium and caladium

The " Feugo" variety of *Anthurium andraeanum* and the " Freida Hemple" variety of caladium was selected as experimental materials. Comparison on the effect of medium formulation and illumination on the induction and regenerating of callus and shoot from the petiole and leaf disk of tissue cultured plantlets. Culture medium: A. Callus induction medium: T0-t：1/8 MS、BA 1ppm、2,4-D 0.08ppm; T0-p：1/2 MS、TDZ 0.5ppm, and B. Shoot regeneration medium: T1-t：1/8 MS、BA 1ppm and T1-p：1/2 MS、BA 0.1ppm. Illumination treatments: light (L, 16/8hr) and dark (D) treatments. Experiments were conducted in combination of medium and illumination treatments.

Regeneration and pigment gene transformation in chrysanthemum and caladium

Plasmids of pigment genes, CHI, DFR constructed in pCambia series vector with various selection markers (cefatoxime, hygromycin), reporter genes (GFP, GUS) in either sense or anti- sense direction were used for transformation. Agrobacterium, EHA105 or LBA4404 strain, transformed with plasmid of pigment gene was prepared according to Sherman et al. (1998) with modifications. Explants (stem segment or leaf) pre-cultured for five days were used for transformation. Explants were dipped with freshly prepared agrobacterium (OD_{600}=0.6) were cultured in regeneration medium (MS medium with 1mg/L BA, 0.1 mg/L NAA) with 100μM acetosyringone at 22℃ for two days in the dark. After three times washing with sterilized water, the explants were washed with 500ppm Cefatoxime then cultured in the regeneration medium (containing 500ppm Cefatoxime) in the dark for four days. Transformed plants were screened in the selection medium containing Cefatoxime 500ppm and hygromycin 15ppm and cultured at 25℃ in the light and sub-cultured every two weeks. Green shoot emerged from the wounded region of explant could be observed at about 60 days after culture.

Analysis of flower pigments by HPLC

Petals of plant materials were harvested and anthocyanin pigments were extracted with 1% HCl-MeOH followed with 1.5 N HCl and quantified by spectrophotometry at A_{530} then, calculated by C=A/38000 L/mole×1.2 cm /molar. The HPLC qualification was conducted at mobile phase：0.425% phosphoric acid：MeOH = 1：1；wave length：530 nm；flow rate：1 ml/min （0.425% phosphoric acid：MeOH = 1：1） using a Inertsil ODS-3 column（5 μm, 4.6×250 mm, GL Sciences Co., Japan）in a Waters HPLC (USA) as described by Reddy et al. (1995).

RESULTS AND DISCUSSION

Three CHI cDNAs were cloned from soybean

Three CHI cDNAs, CHI1, CHI2, and CHI3, were screened from the cDNA library made from young (<100mg) seed coats of CRWD variety (Fig.1, 2), however, the Southern analysis (data not shown) showed that only one copy of CHI gene was

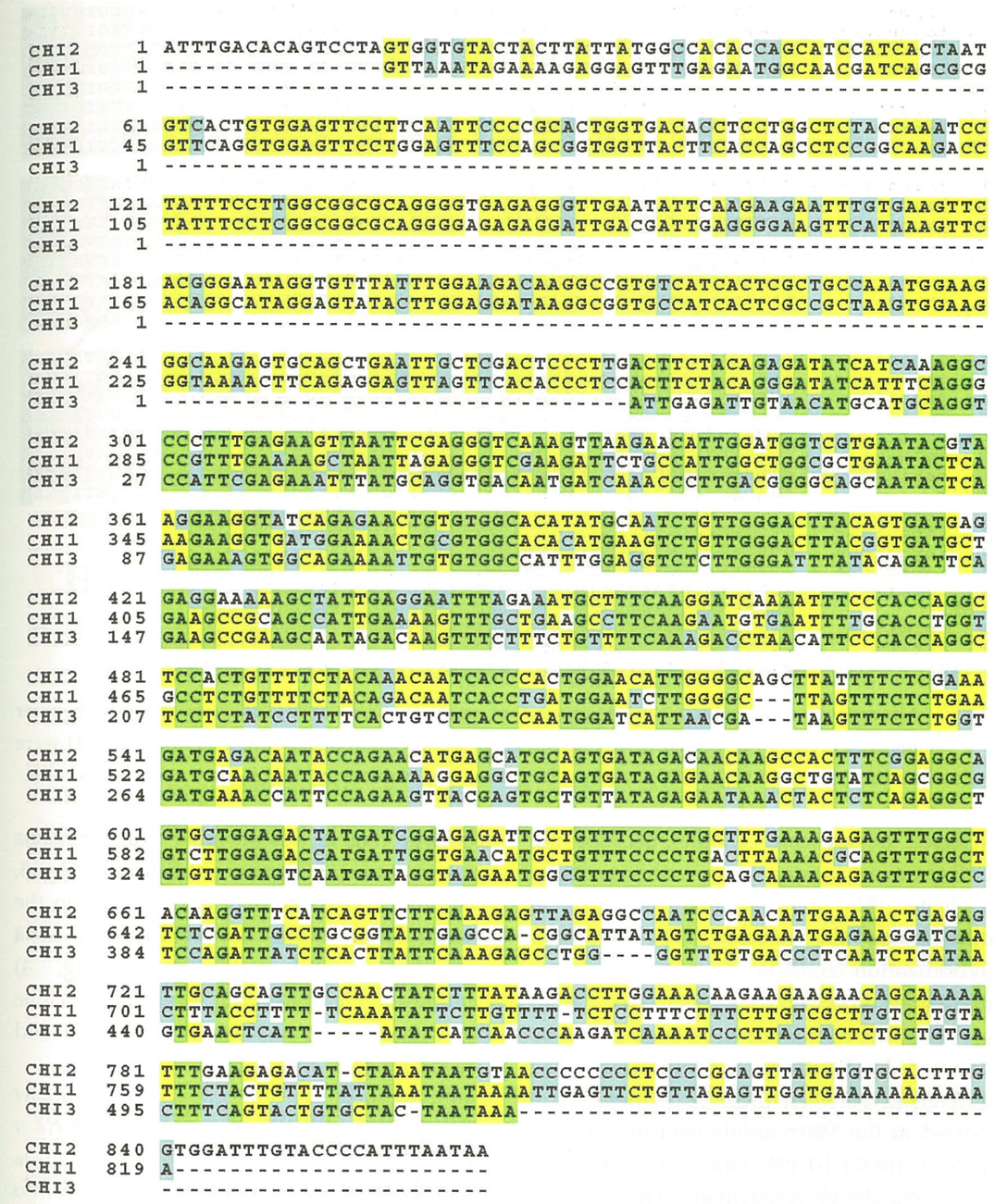

Fig. 1. Alignment of three soybean *CHI* gene sequences. Complete sequences of soybean CHI1, CHI2 and partial sequences (3′ end) of CHI3 are aligned using CLUSTALW program. The conserved regions are highlighted with green color.

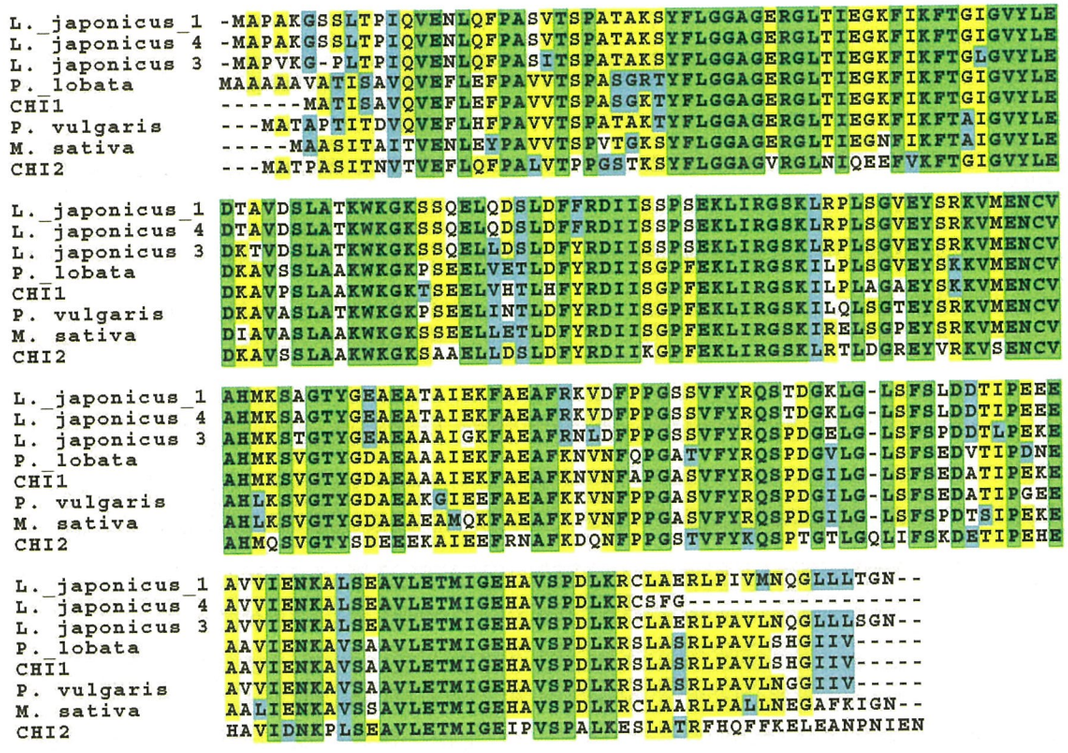

Fig. 2. Alignment of the amino acid sequences of soybean *CHI1* and *CHI2* sequences with other legume *CHI*s. Type II CHI from legume species (accession number as listed in Table 1) were aligned using CLUSTALW program. The conserved regions are highlighted with green color.

detected in soybean genome. Because the CHI genes are located on soybean genome as a cluster (Fig. 4) it is reasonable that to differentiate the difference by Southern hybridization could be difficult. This is the first paper report on CHI genes cloned from soybean. The nucleotide difference cannot be detected by Southern hybridization. The differences between these two proteins are located at the 184th amino acid in which A and T is for CHI-1 (S4 clone) (AF276302) and CHI-2 (S2 clone), respectively. These results suggested that the CHI gene in soybean genome may exit as a duplicate gene therefore the cloning of CHI gene will answer the question. By genomic library screening, a genomic clone, *CHI* 1, with 8,520bp (Fig. 2) was obtained which contains 4 exons and 3 introns as shown in Fig. 3. Another CHI gene may also located on the same chromosome of soybean but in a reverse direction as *CHI* 1 (Fig. 4) unfortunately, this is not a full length clone and need to be completed to provide a solid result for gene structure.

Molecular cloning of CHI2 genes by genome walking

A technique, genome walking, developed by BD Biosciences and the GenomeWalker Kit, was applied for cloning the flanking sequences of CHI genes from soybean genome. A primer, 5′-TGCTCGACTCCCTTGACTTCTACAG-3′,

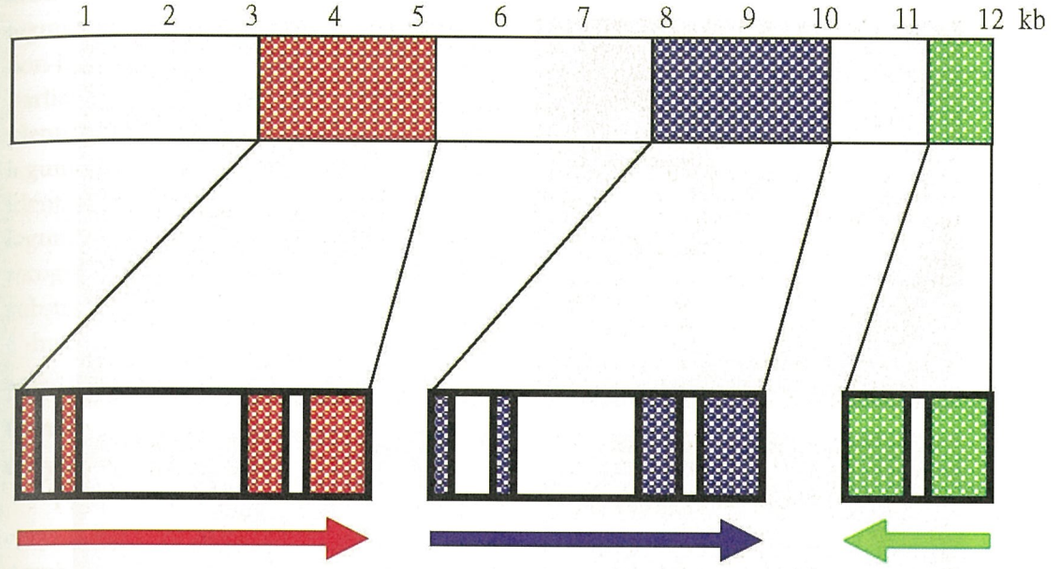

Fig. 3. Gene structures of soybean *CHI* genes. Three colored blocks represent different *CHI* genes of soybean. The red , blue and green boxes represent for the *CHI1*, *CHI2* and *CHI3*, respectively. Colored boxes represent exons while the empty boxes represent for introns. The arrow represents direction of transcription of each *CHI*.

was designed for the first round of genome walking. Two DNA fragments were amplified from *Dra* I and *Pvu* II libraries constructed from restriction enzyme digested genomic DNA of UC9 (*iRT* genotype). To amplify the entire *CHI2* gene, a new primer, 5′-TTGCATGCCTTG-AAGATCTGATAAC-3′, was designed for second round of genome walking. A specific DNA. fragment (3.0 kb) amplified from *Eco*R V library was cloned and sequenced to complete the *CHI2* genomic sequences. A partial 3′ end gene sequences of the third CHI gene, *CHI3*, was found in the opposite direction by BLAST analysis (Fig. 5). The amino acid sequences *CHI1* and *CHI2* are

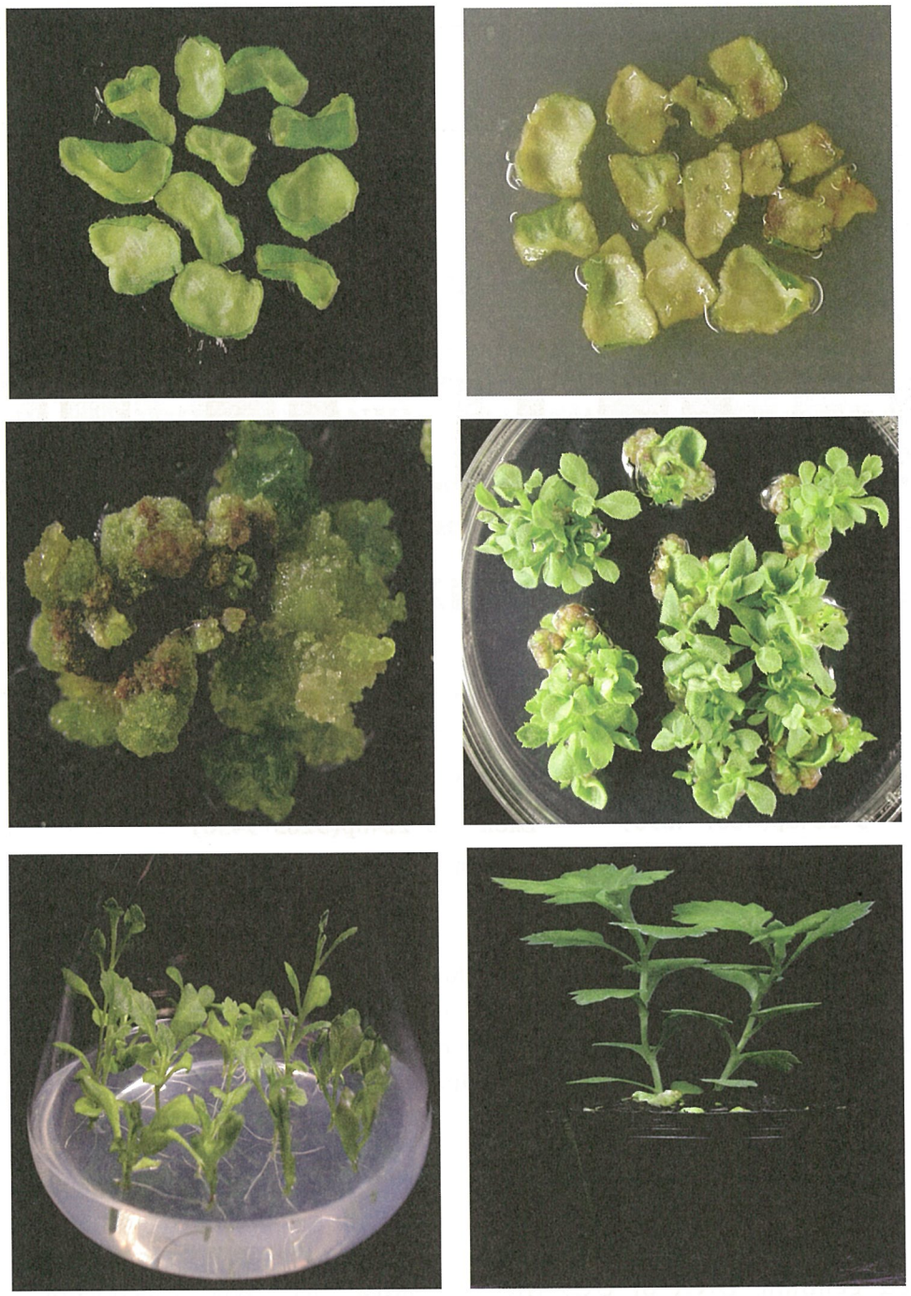

Fig. 4. Flow chart of transgenic chrysanthemum.(A)pre-culture of explant;(B)co-cultivation with agrobacterium;(C)callus induction and regeneration on hygromycin medium;(D)shooting on hygromycin regeneration medium;(E) rooting of regeneration shots on hygromycin selection medium;(F) transgenic plants at six months after transformation.

around 70% identical to the Type II CHI of leguminous plants and have low identity to non-leguminous plants (Table 1 and 2). The partial cDNA sequences of *CHI3* show high identity (about 70%) to type I CHI of non-leguminous plants and with only 50% identities while compared to type II CHI of leguminous plants Type I CHI only recognizes 6'-hydroxylasechalcone as a substrate, however, type II CHI catalyzes both 6'-hydroxychalcone and 6'-deoxychalcone.

New flower color developed by transformation of soybean CHI gene to chrysanthemum

Pigment genes were transferred to chrysanthemum and caladium by *Agrobacterium*-mediated method, putative transgenic plants were screened in the selection media containing proper antibiotics and followed with PCR to obtain transgenic plants with CHI gene. An efficient transformation system has been established in this proposal as demonstrated in the Fig. 5. Though there exits cultivar specific problems in the transformation of chrysanthemum, our system can be modified for many commercial varieties. With this system, the transformation process can be completedwithin nine months to obtain flowering plants in crysanthemum. We have obtained 28 different transgenic anti-sense CHI chrysanthemum plants (Fig. 4). After antibiotic screening, they were further screened for both GUS and CHI genes by PCR. The flowers colors were expected to be yellow if the expression of CHI gene was inhibited by transgene however, the flowers of transgenic mum show only light yellow at the early stage of flower development (Fig. 4). The explanations for this observation is that: 1.there may not have narigenin chalcone accumulated in the transgenic plants because of lack of CHS in this white color variety, 2. the soybean CHI gene may not be able to complete block the expression of CHI gene in chrysanthemum due to the less similarity in DNA sequences. However, further experiment is required to answer this observation.

Interesting, several transgenic plants (Fig. 5) with the over-expression CHI construct show altered color from red control to yellow suggesting that the expression of endogenous CHI gene is suppressed by the transformed soybean CHI gene as reported by Meyer et al., (1987) when transferred the CHS gene of maize to petunia. According to HPLC analysis, the original Red Beauty flowers contains only cyaniding and show red color, however, the transgenic CHI plant contains lutein, β -carotene of carotenoid biosynthesis pathway but not cyanidin and show yellow color suggesting that the endogenous anthocyanin biosynthesis pathway has been blocked.

As we know, our experiment provides the first example of flower color engineering through transformation of a heterogeneous CHI gene from soybean. Besides, we also demonstrate that plant metabolic pathway could be altered through transformation of a similar gene differs in gene function.

Similarly, soybean CHI1 genes were also transferred to caladium by *Agrobacterium*-mediated method, putative transgenic plants were screened in the selection media containing proper antibiotics as previously described or can be subjected to fluorescent microscope detection for selection marker 'GFP' expression. An agrobacterium-mediated transformation system has established for caladium with high efficiency. Transgenic plants with anti-sense CHI gene show three types of leaf colors as compare

with control. The first type of transgenic caladium show similar color as control plants. The second type shows very light red on their leaves, the third type of transgenic plants shows almost no red pigment accumulated on their leaves (Fig. 6). The pigment biosynthesis on the leaves of the type II and type III were inhibited by various levels of anti-sense CHI gene of soybean

Wild type Transgenic CHI Wild type Transgenic CHI

Fig. 5. Flowers of transgenic CHI chrysanthemum show yellow color that accumulates lutein and carotene instead the red cyanidin of the wild type (Red Beauty).

Fig. 6. The leaves color of control (upper most) and transgenic anti-sense CHI caladium plants.

and show no red pigment accumulated, no yellow color was observed on leaves as we have expected. A reasonable explanation is that the yellow color of narigenin chalcone was covered by green chlorophyll therefore, yellow color cannot be visible. Biochemical analysis will provide reasonable explanation for this observation. In the future, proper variety will be chosen for pigment transformation to obtain yellow or blue-purple caladium. As we know, this is the first report on leaf color engineering on caladium and ornamental leaf plants. Because, all the mass propagation systems for both chrysanthemum and caladium have well established, any good transgenic plants can be commercialized within one year.

ACKNOWLEDGEMENTS

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Kinetic Studies of Lycopene Isomerization in a Food Model System at Gastric pH

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ABSTRACT

A semi-preparative HPLC method was developed in order to isolate and purify the 13-*cis* lycopene isomer in tomato-based materials. The result was compared with the naturally-predominant all-*trans* lycopene isomer, in terms of stability to gastric pH at physiological temperature in a tributyrin model system. Kinetic experiments confirmed that lycopene isomerization is a reversible reaction and under these conditions the all-*trans* isomer is more stable than the 13-*cis* isomer. In addition, it was found that at gastric pH 13-*cis* lycopene would predominantly isomerize to the all-*trans* form rather than undergo oxidation/breakdown. A simulation based on the rate constants calculated in the kinetic study indicated that at gastric pH the lycopene isomeric distribution aimed towards an equilibrium characterized by approx 16% 13-*cis*, 16% 9-*cis* and 68% all-*trans* lycopene. This study suggests that pH-driven isomerization in the stomach is at least partially responsible for the relatively high *cis* lycopene proportion found *in vivo*.

Key words: Lycopene, *cis-trans* Isomeri-zation, Kinetics, Gastric pH, Tributyrin.

INTRODUCTION

One of the reasons for the popularity of tomato is its pleasant red color, imparted by lycopene. Lycopene is an acyclic C_{40} non-polar carotenoid found in tomatoes, watermelon, guava, apricots and other natural sources. The molecule is highly unsaturated, the 11 conjugated double bonds being responsible for the ruby red color. Of the theoretical 2^{11} geometrical isomer forms, only 72 are thermodynamically possible [1]. All-*trans* lycopene is the predominant lycopene isomer in nature, representing about 80-97% in tomatoes and related products [2]. In body fluids, however, more that 50% of lycopene was found in various *cis* forms [3], leading to the suggestion that the 50:50 ratio between the *cis* and all-*trans* forms may represent an equilibrium in physiological conditions [4]. Investigators proposed several explanations of this finding, such as the preferential absorption of *cis* lycopene isomers within the intestines [5], or the isomerization of all-*trans* lycopene in the stomach or intestines [6].

The high concentrations of *cis* isomers *in vivo* triggered the hypothesis that they may be more bioavailable and/or more bioactive than the all-*trans* form. Studies of the *cis:trans* isomer ratios in various organs and

tissues of ferrets fed with a lycopene-rich diet [2;4] suggested that the *cis* isomers are indeed more bioavailable than the all-*trans* isomer, possibly due to the higher solubility and/or a preferential incorporation of the *cis* isomers into chilomicrons. In terms of bioactivity, Shi and LeMaguer [7] pointed out that the bioactive potency of *cis* lycopene isomers is different from that of the all-*trans*, because of structural differences. Böhm *et al.* [8] indeed found that three unidentified *cis* isomers had a stronger antioxidant activity than the all-trans. For all these reasons, *cis* lycopene isomers are regarded as having potentially higher health benefits than the all-*trans* isomer.

Lycopene isomerization triggered by the gastric pH is believed to be one of the reasons for the unusually high amounts of *cis* isomers, when compared to the lycopene intake. An *in vitro* study [6] found that gastric pH is able to trigger the isomerization of all-*trans* lycopene to *cis* forms after an exposure as low as 3 minutes. However, an *in vivo* investigation of the *cis:trans* lycopene ratio in human subjects fed intragastrically [9] found that it did not significantly vary in the stomach during digestion, suggesting there is little isomerization, if any. These conflicting results leave open the question whether low pH-driven isomerization indeed plays a role in the large proportion of *cis* lycopene isomers found *in vivo*. On the other hand, no information was found on the relative stability of the *cis* forms vs. the all-*trans* under low pH conditions, and the quantification of the relative stability would contribute to the better understanding of the *in vivo* changes. Since the *cis* lycopene isomers are not commercially available, the comparative investigation of *cis-trans* lycopene isomers in processing or physiological conditions is typically performed by monitoring the changes of all-*trans* or mixtures of lycopene isomers. The drawback of this approach is that the system includes several *cis* forms, in very small amounts, which makes the comparison less accurate.

The objectives of this study were to isolate and purify the most abundant *cis* lycopene isomer from tomato-based sources, and to compare its stability to the stability of the all-*trans* form, when subjected to gastric pH at physiological temperature in a tributyrin model system. The purified compounds were used to collect kinetic data enabling the calculation of reaction rate constants for the isomerization reactions undergone by the predominating isomers. Thus, this study attempts to improve the understanding of pH-driven lycopene isomerization *in vivo*, enabling the prediction of the extent of isomerization based on gastric residence time.

MATERIALS AND METHODS

Preparation of the lycopene stock

The procedure was derived from procedures reported in the literature [10 -12], and was performed in aluminum foil-wrapped glassware or, when not possible, in dim light. All reagents were purchased from Fisher Scientific (Fairlawn, NJ), and solvents were HPLC grade.

Tomatoes for fresh consumption, purchased from a local supermarket (Shop-Rite, Elizabeth, NJ), were used as source for preparing all-*trans* lycopene.

Quantification of lycopene isomers

The lycopene isomers were quantified by analytical HPLC, using a dedicated 150×4.6mm i.d., 3 µm, Develosil RP-Aqueous C_{30} column (Phenomenex Inc., Torrance, CA), protected by a C_{18} SecurityGuard column, both kept at 25°C. 50 µL of material were injected, using a loop of 50 µL (Rheodyne L.P., Rohnert Park, CA). The HPLC method used to quantify lycopene isomers was derived from the method reported by Yeum *et al.* [14], modified (Table 1) in order to improve resolution while decreasing the resolution time. Solvent A was a mixture of methanol:MTBE:water (83:15:2, v/v/v), and solvent B was methanol:MTBE:water (18:80:2, v/v/v). β-8'-apo-carotenal was used as internal standard [15], both for building the calibration curve and in the experimental injections.

The HPLC equipment consists of a 600E solvent delivery system and 991 photodiode array detector (Waters Corp., Milford, MA) and a computer running the dedicated Waters 991 PDA software. For faster processing, the signal at 471 nm, the absorption maxima for lycopene [13], was exported via an analog output channel to a second computer running the Diamir chromatography software (JMBS, Bear, DE).

Preparation of the *cis* lycopene isomer

To improve the *cis* isomers proportion, isomerization of stock lycopene was forced by placing aliquots of the stock (concentration approx. 120 mg/L) in a 1.5 mL brown, septum-closed GC vial (National Scientific, Duluth, GA), and pre-heating the vial by submersion in an oil bath for 5 min at 85 ℃ (oil temperature). All-*trans* and the predominating *cis* lycopene isomer were isolated from the pre-heated stock by cooling it down and directly injecting the concentrated stock in the HPLC, in order to increase the concentration of the isomers collected [16].

The HPLC system used for the preparation of larger amounts of lycopene isomers was the system described above, except it used a 250×10 mm i.d., 5 µm, Develosil RP-Aqueous C30 semi-preparative column protected by a semi-preparative scale C18 SecurityGuard column (both provided by Phenomenex Inc., Torrance, CA), both kept at 25°C.

Identification of the lycopene isomers prepared

The fractions carrying lycopene isomers were tentatively identified spectrophotometrically: 1 mL of each fraction was placed in a quartz cuvette (Fisher Scientific, Fairlawn, NJ) and its absorption spectrum was read between 300 and 550 nm, at a rate of 100 nm/min, using a U 3110 UV/Vis spectrophotometer (Hitachi Instruments, San Jose, CA). The fractions containing lycopene isomers were further

Table 1. Gradient scheme for the analytical HPLC method. Solvents were: A= methanol: MTBE:water 83:15:2 (v/v/v); B = methanol:MTBE:water 8:90:2 (v/v/v).

Time, min	Flow, ml/min	%A	%B	Type
0	1	90	10	
4	1	45	55	linear
8	1	5	95	flat
16	1	5	95	linear
18	1	90	10	linear

analyzed with the analytical HPLC method, which allowed pooling together neighboring fractions showing the same pattern.

The structural identity of the fractions containing the main two isomers was confirmed using ^{1}H-NMR. For this purpose, the solvent was removed by flushing with nitrogen, in the dark and on ice, and then the dried residue was re-dissolved in deuterated chloroform [17]. The samples were analyzed using a Varian 400 UNITYINOVA NMR spectrophotometer with the VNMR version 6.1B (Varian NMR Systems, Palo Alto, CA) software package, at room temperature. The spectra obtained were compared with published lycopene isomers NMR spectra [18], and the identity of isomers was elucidated by comparing the value of the spectroscopic shifts.

Kinetic studies of lycopene stability in a model system, at gastric pH

Lycopene is solid and hydrophobic, so a hydrophobic model system able to dissolve lycopene was necessary. Criteria such as liquid state, ability to dissolve lycopene, stability at temperatures above 100 °C, lack of rancidity or oxidation and relatively low viscosity were used to select the system. The system of choice was tributyrin, a liquid triglyceride having the residues of the saturated butyric acid esterified to glycerol, found in small amounts in butter.

The experiment used a solution of 13-*cis* lycopene in tributyrin (concentration of 19.8 mg/L) and a solution of all-*trans* lycopene in tributyrin (concentration of 20.5 mg/L). 1 mL of isomer solution was mixed with 2 mL simulated gastric juice (composition: 0.7% hydrochloric acid, 0.2% sodium chloride, 1.07 ppm pepsin in distilled water; pH 1.5; Fisher Scientific, Fairlawn, NJ) and 5 µL Tween-20. The emulsion was prepared by sonication on ice in an XL2020 instrument (Heat Systems Inc., Farmingdale, NY) set at level 5, using six 10 s steps alternating with 10 s rest. The simulated gastric juice (SGJ) was pre-heated at 37℃, and the temperature of the emulsion after preparation did not exceed 42 ℃ in any of the samples. An aliquot of the emulsion was stored at room temperature to check the stability, and 48h after preparation did not show any phase separation.

Aliquots of the emulsions were placed in septum-closed brown GC vials and immersed in an oil bath preheated at 37±1 °C. Each 30 min, 50 µL of each sample were injected in the HPLC, and the concentration of each lycopene isomer was quantified using the analytical method described above, assuming the same specific absorption coefficient for both isomers [8]. The experiments were performed in two replicates, and lasted for 6 h, after which peaks were below the limit of quantification.

RESULTS AND DISCUSSION

Preparation and identification of the lycopene isomers prepared

Comparison and spiking tests of the lycopene stock with authentic all-*trans* lycopene (Sigma-Aldrich, St. Louis, MO) showed that the lycopene stock mostly consisted of all-*trans* lycopene. HPLC quantification found that the all-*trans* isomer accounted for about 90% of the lycopene stock.

Chromatograms of the lycopene stock showed four main peaks (Fig. 1). While spiking experiments showed that peak I belongs to the internal standard and peak IV belongs to all-*trans* lycopene, peaks II and III were tentatively identified as 13-*cis* and 9-*cis*

lycopene, by comparison with the chromatograms published by Schierle *et al.* [19]. The quantification of these *cis* isomers was performed assuming the same specific absorption coefficient as the one calculated from the calibration curve for all-*trans* lycopene–this assumption was used previously by Böhm *et al.* [8] and Ishida *et al.* [20]. The Q-ratios for the tentatively-identified *cis* lycopene isomers were calculated as the ratio between the areas of peaks II or III and the area of peak IV.

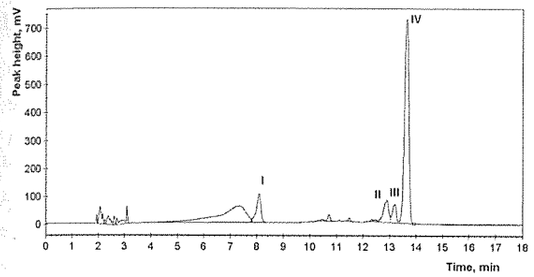


Fig. 1. Chromatogram of the lycopene stock. Peaks are: I–internal standard (β-8'-apo-carotenal); II–13-*cis* lycopene; III–9-cis lycopene; IV–all-*trans* lycopene.

The fractions collected using the preparative method were screened spectrophotometrically, and the ones showing higher color intensities were further analyzed by HPLC and ^{1}H-NMR (data not shown). While the all-*trans* fraction appeared to be close to purity, the assumed 13-*cis* fraction still had small amounts of all-*trans* and 9-*cis* isomers, and the assumed 9-*cis* fraction was even less pure. Possible causes for these residues could be a partial co-elution of *cis-trans* isomers (reported also by Böhm *et al.* [8]) or generation of the all-*trans* form as an artifact between separation and storage in the freezer. However, the very short exposure to light and room temperature makes the second possible cause rather improbable. For purity reasons, the fractions collected were the ones corresponding to all-*trans* and 13-*cis* lycopene.

The ultimate proof was provided by the NMR spectra of the assumed lycopene isomer preparations. The hydrogen atoms involved bound to the polyene chain carbons have chemical shifts between 5 and 7 ppm [17, 18], so this particular zone of the NMR spectrum can be used to identify the isomer. The chemical shifts found experimentally for the two preparations analyzed showed very good match with the NMR data reported by Hengartner *et al.* [18], both for the all-*trans* and 13-*cis* lycopene, which confirmed the identity of the two fractions investigated by NMR.

While the purity of the *cis* lycopene fractions collected using this method is critical, the yield is also very important for the feasibility of further experiments. In order to quantify the amount of fractions prepared, about 1 mL of all-*trans* stock preparation (concentration of 323.6 mg/L) was used to prepare *cis* forms as described. Based on the assumption of the same specific absorption coefficient, about 48 µg of 13-*cis* and about 160 µg of all-*trans* lycopene were prepared. A balance of lycopene found that in the pre-heating process less than 30% of the initial all-*trans* lycopene broke down, while 71% was found in the preparations collected.

Kinetic studies of lycopene stability in a model system, at gastric pH.

The concentrations of each lycopene isomer in time are shown in Fig. 2. In both cases, the isomer that predominated in the initial material underwent a steady loss with time, while the other isomer accumulated up to a maximum, after which the concentration

decreased. This result confirms that the pH-driven isomerization is a reversible reaction: the all-*trans* isomer was lost with formation of 13-*cis* form (Fig. 2A), while 13-*cis* lycopene was lost with formation of the all-*trans* isomer (Fig. 2B). This result is easier to visualize when looking at the Q-ratio (Fig. 3): the 13-*cis* Q-ratio showed an initial large drop when starting from 13-*cis* lycopene, but the curve flattened rather quickly. When starting from all-*trans* lycopene the Q-ratio steadily increased, indicating a continuous accumulation of the 13-*cis* isomer.

The total lycopene concentration in Fig. 2 was calculated by summing the concentrations of the all-*trans*, 13-*cis* and 9-*cis* isomers; the other isomers were probably negligible as new peaks did not appear in the chromatograms. The curves for total lycopene had similar slopes in Fig. 2A and 2B, suggesting that overall lycopene was lost to a similar extent in both cases, and confirming that low pH causes not only lycopene isomerization, but also breakdown. This was in fact expected, since acids such as nitric or trichloroacetic are known to initiate degradation of carotenoids in foods by isomerization or oxidation: Mortensen and Skibsted [22] reported that carotenoids are protonated slowly by such acids, by a mechanism still largely unknown. It was also reported that carotenoids can react with Lewis acids (e.g. I_2), generating species that absorb in the same area as carotenoids radicals [22].

When the experiment started with all-*trans* lycopene (Fig. 2A), the all-*trans* concentration dropped rather quickly, as shown by the steep slope. The slope of the curve corresponding to 13-*cis* lycopene in Figure 2B was comparatively less steep, leading to the hypothesis that the 13-*cis* isomer may be more stable than the all-*trans* at low pH. This hypothesis justified the goal of calculating the kinetic reaction rate constants for the isomerization reactions.

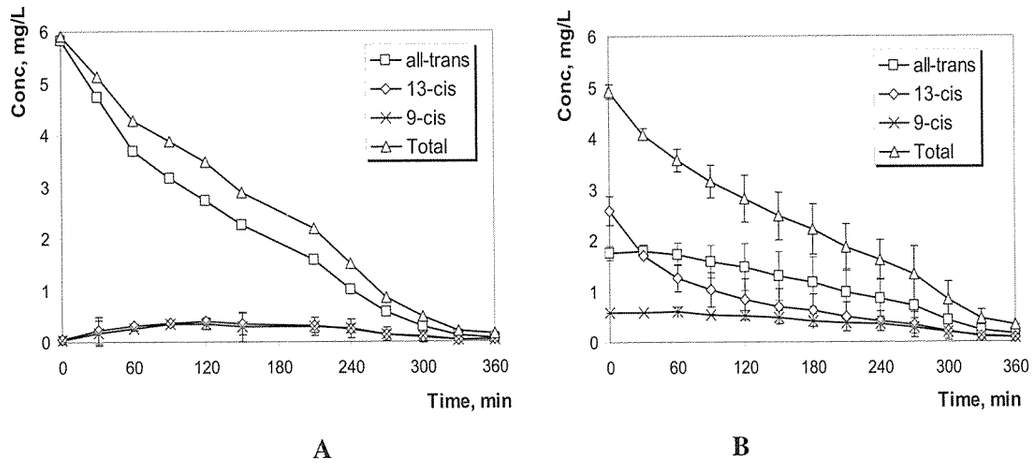


Fig. 2. Concentrations of lycopene isomers during exposure of all-*trans* and 13-*cis* lycopene to gastric pH for 6 hours. A. all-*trans* fraction; B. 13- *cis* Fraction.

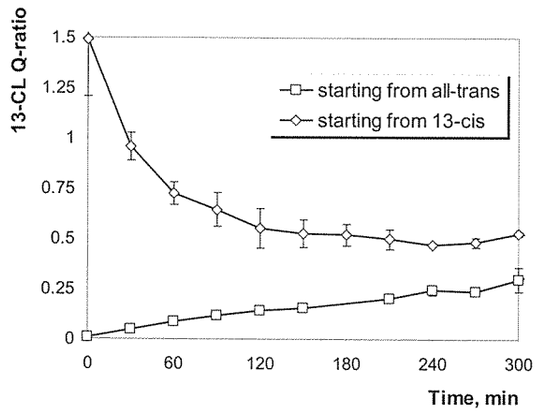


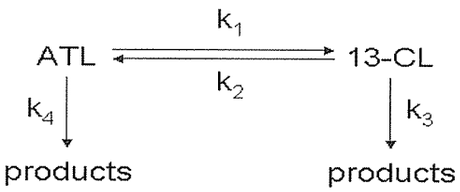
Fig. 3. 13-*cis* lycopene Q-ratios during exposure of all-*trans* and 13-*cis* lycopene to gastric pH for 6 hours.

The curves of 13-*cis* and 9-*cis* lycopene in Fig. 2A were very similar, indicating that at low pH both isomers were formed to a similar extent. However, their concentrations were low when compared to Figure 2B, suggesting that at low pH the predominating reaction for the all-*trans* isomer would be breakdown, not isomerization. Interestingly, when starting from 13-*cis* lycopene (Fig. 2B), both all-*trans* and 9-*cis* lycopene curves had a rather flat appearance. Since in such conditions the all-*trans* isomer was lost rather quickly (Fig. 2A), its generation by isomerization of 13-*cis* lycopene must have been large enough to partially compensate the loss and lead to the small overall decrease in Figure 2B. Therefore, at gastric pH, isomerization of 13-*cis* lycopene to all-*trans* must be quite significant.

In summary, the experimental data suggests that at gastric pH 13-*cis* lycopene was lost to a lower extent than all-*trans* lycopene, all-*trans* lycopene had a tendency to breakdown rather than isomerize, while the isomerization of 13-*cis* lycopene to all-*trans* was more intense than its breakdown. These hypotheses were verified by performing the kinetic study described above.

Calculation of reaction rate constants for the isomerization of all-*trans* and 13-*cis* lycopene at gastric pH

The data acquired in the kinetic study was used to calculate the reaction rate constants for the isomerization of 13-*cis* and all-*trans* lycopene. With the exception of the all-*trans* and 13-*cis*, the other lycopene isomers were not individually considered. Since their concentrations were much lower, for this study they were included in the generic term "products", together with breakdown products. Therefore, the pH-driven isomerization process was considered to occur according to the following schematic:



The other assumption was that the

reactions followed first order kinetics. This assumption is based on the numerous studies reporting first order kinetics for degradation of carotenoids in various environments [23-25], and was necessary in order to build the mathematical model describing the reactions in Fig. 1.

If the reactions in the schematic above followed first order kinetics, then the concentrations of all-*trans* and 13-*cis* lycopene at any given time are given by the equations:

$$\begin{cases} \frac{d[\mathrm{ATL}]}{dt} = -k_1 \cdot [\mathrm{ATL}] - k_4 \cdot [\mathrm{ATL}] + k_2 \cdot [13-\mathrm{CL}] & (1) \\ \frac{d[13-\mathrm{CL}]}{dt} = k_1 \cdot [\mathrm{ATL}] - k_2 \cdot [\mathrm{CL}] - k_3 \cdot [13-\mathrm{CL}] & (2) \end{cases}$$

where the notation [*X*] indicates the concentration of species *X*. The reaction rate constants k_1 and k_2 characterize the reversible, pH-driven isomerization of all-*trans* lycopene to 13-*cis*.

The system of equations (1) and (2) can be used to calculate the reaction rate constants k_1, k_2, k_3 and k_4. When starting from all-*trans* lycopene the 13-*cis* concentration reached a maximum (Fig. 2A), and conversely when starting from 13-*cis* lycopene the all-*trans* concentration reached a maximum (Fig. 2B). At these maximum points, the derivatives of the concentration (left term in equations (1) and (2)) are zero. This observation, also used in a more complex approach [25], allows solving the system of equations (1) and (2) numerically. When starting from all-*trans* lycopene, after rearrangements, equation (2) becomes:

$$\frac{d[13-CL]}{dt} = k_1 \cdot ([ATL] - K \cdot [13-CL]) \quad (3)$$

where $K = [\mathrm{ATL}]_M / [\mathrm{13\text{-}CL}]_M$ is the isomers concentrations ratio at the maximum point. Therefore, the value of k_1 can be calculated by linear regression, using equation (3). Similarly, when starting from 13-*cis* lycopene, the value of k_2 can be calculated from the linear regression of the equation:

$$\frac{d[\mathrm{ATL}]}{dt} = k_2 \cdot \left([13\text{-}\mathrm{CL}] - K' \cdot [\mathrm{ATL}]\right) \quad (4)$$

where $K' = [\mathrm{13\text{-}CL}]_M / [\mathrm{ATL}]_M$.

The value of each derivative in equations (3) and (4) was approximated with the slope for that particular time interval, taken at the midpoint. For example, for 13-*cis* lycopene:

$$\frac{d[13-CL]}{dt} \cong \frac{[13-CL]_{t_2} - [13-CL]_{t_1}}{t_2 - t_1} \quad (5)$$

where t_1 is the time at the beginning of the interval considered, and $t_2 = t_1 + 30$ min is the time corresponding to the end of the interval. The concentrations of all-*trans* and 13-*cis* lycopene at the midpoint of each time interval were calculated with:

$$[\mathrm{ATL}] = \frac{[\mathrm{ATL}]_{t_1} + [\mathrm{ATL}]_{t_2}}{2} \text{ and } [13\text{-}\mathrm{CL}] = \frac{[13\text{-}\mathrm{CL}]_{t_1} + [13\text{-}\mathrm{CL}]_{t_2}}{2} \quad (6)$$

In order to improve the accuracy of the result, the calculation used each data point, not the averages [26].

The reaction rate constant for all-*trans* lycopene isomerization with formation of the 13-*cis* isomer, obtained from the linear regression, was 0.0016/min. The coefficient of determination r^2 was about 0.81, indicating a strong correlation. Since the number of experimental points is 22, the number of degrees of liberty for the correlation analysis is 20, therefore with $\alpha = 0.05$ the critical r value is 0.423 [27]. This suggests that the linear correlation above is highly significant.

For the isomerization of 13-*cis* lycopene to all-*trans*, the value of k_2 calculated is 0.0053/min, but with much weaker correlation ($r^2 = 0.19$). However, the critical value of r for $\alpha = 0.05$ and 20 degrees of liberty is 0.423 [27], so the correlation is still significant. It should be noted that the value of k_2 (0.0053/min) is higher than the one of k_1 (0.0016/min). A higher k_2 value indicates that the isomerization of 13-*cis* lycopene back to all-*trans* is a faster reaction that the direct isomerization of all-*trans* lycopene to 13-*cis*.

Once the reaction rate constants k_1 and k_2 for the isomerization reactions were found, it was possible to calculate the reaction rates k_3 and k_4 for the breakdown reactions. The value of k_3 can be calculated using equation (2), from the linear regression between $k_1 \cdot [\mathrm{ATL}] - k_2 \cdot [\mathrm{ATL}] - d[13\text{-CL}]/dt$ and [13-CL]. The value of k_3 found by this calculation was 0.0022/min, but the very poor correlation coefficient (0.006) indicates it was not significant. The value of k_4 was similarly calculated from equation (1), using the linear regression between $k_2 \cdot [13\text{-CL}] - k_1 \cdot [13\text{-CL}] - d[\mathrm{ATL}]/dt$ and [ATL]. The calculated value was 0.0004/min, but again with a poor and non-significant correlation coefficient (0.06).

The fact that the correlations for calculating k_3 and k_4 are not significant was in fact expected, since the model used to quantify the kinetics of pH-triggered isomerization and breakdown is over-simplified. In fact, there were many other reactions involving both all-*trans* and 13-*cis* lycopene, both isomerizations and oxidations with formation of smaller products, and they most likely occurred at various rates and possibly following different kinetics orders. However, this simplified model allowed us to obtain quantitative information about the kinetics of the reversible isomerization of all-*trans* to 13-*cis* lycopene.

The reaction rate constants confirm that all-*trans* lycopene is more stable than 13-*cis* lycopene at low pH. The isomerization of all-*trans* lycopene to 13-*cis* has $k_1 = 0.0016$/min, while the isomerization of 13-*cis* lycopene to all-*trans* has $k_2 = 0.0053$/min, being 3.5 times faster. The values of k_3 and k_4 were not significant, but if taken as an order of magnitude then the 13-*cis* isomer undergoes breakdown about 5 times faster than the all-*trans* isomer. For 13-*cis* lycopene the isomerization to all-*trans* had a reaction rate $k_2 = 0.0053$/min, while the breakdown reaction constant appeared at least 2 times lower, which indicates that the hypothesis that at low pH 13-*cis* lycopene would rather isomerize to all-*trans* than breakdown is probably valid.

The question whether at low pH all-*trans* lycopene would rather isomerize than breakdown is not clearly answered by this study. Since $k_1 = 0.0016$/min, at low pH all-*trans* lycopene would isomerize easily to 13-*cis*. However, the other breakdown or isomerization processes in which all-*trans* lycopene participates could not be modeled by a single first-order reaction, so kinetic constants to characterize these other processes could not be generated for comparison. It may be possible to get a clear answer to this question if considering the 9-*cis* lycopene levels and adding a third equation describing the variation of its concentration, similar to equations (1) and (2). However, this requires purification of the 9-*cis* isomer (which is difficult as it elutes much closer to all-*trans* lycopene) and performing the same study starting with 9-*cis* lycopene. Besides doubling the amount of experimental work, the mathematical analysis of the model becomes very complex.

An interesting question is: why, when starting from the all-*trans* isomer, a more

significant accumulation of *cis* isomers was not found? This is largely an effect of the magnitude of k_1 and k_2: since $k_2 >> k_1$, any *cis* lycopene formed would quickly isomerize back to all-*trans*. To test this hypothesis, the model described by the schematic above and reaction rate constants $k_1..k_4$ were used to simulate the behavior of lycopene, in two scenarios: starting with 100% all-*trans* lycopene; and with 100% 13-*cis* lycopene respectively. The analysis was done incrementally using Excel (Microsoft, Redmond, WA), the concentrations of all-*trans* and 13-*cis* lycopene being calculated using the equations:

$$\begin{cases} [ATL]_{t+1} = [ATL]_t - (k_1 + k_4) \cdot [ATL]_t \cdot \Delta t + k_2 \cdot [CL]_t \cdot \Delta t & (7) \\ [CL]_{t+1} = [CL]_t + k_1 \cdot [ATL]_t \cdot \Delta t - (k_2 + k_3) \cdot [CL]_t \cdot \Delta t & (8) \end{cases}$$

where the length of the increment was Δt = 5 min. The result of this simulation is shown in Figure 4, and it indicates that when starting from any isomer the other isomer would reach a maximum. This simulation answers the question above: indeed when starting with all-*trans* lycopene a relatively small concentration of 13-*cis* isomer is found, while when starting with 13-*cis* lycopene a relatively higher all-*trans* isomer concentration is found at the maximum. An interesting result was obtained when plotting the Q-ratio for 13-*cis* lycopene (Fig. 4, secondary *y* axis): the simulation indicates that in both cases it plateaus to 0.236, so the model suggests that the system aims to reach a dynamic equilibrium. The isomeric distribution of lycopene at this particular point can be calculated assuming that both 13-*cis* and 9-*cis* lycopene, the main isomers detected, are formed at about the same extent (as indicated by Fig. 2A). In this case, a simple algebraic calculation suggests that the equilibrium lycopene isomeric distribution at gastric pH consists of approx. 16% 13-*cis*, 16% 9-*cis* and 68% all-*trans* lycopene.

This interesting result may put in a new light the fact that in body fluids more than 50% of lycopene was in *cis* forms [5]. There is some controversy regarding the extent of lycopene isomerization triggered by gastric pH: Re *et al.* [6], in an *in vitro* study, showed that gastric pH is able to trigger the isomerization of all-*trans* lycopene to *cis* forms, while Tyssandier *et al.* [9] showed using *in vivo* data that there is no significant *cis-trans* lycopene isomerization in the stomach. The data above suggests that pH-driven isomerization is responsible at least partially for the high *cis* lycopene proportion found *in vivo*. One could then hypothesize that the reason for the higher *cis* concentration in the body is that *cis* isomers are generated in the stomach and probably better absorbed in the intestines because of the more advantageous shapes/sizes of the molecules [2], and the mechanisms of transport from stomach to the various destinations inside the body may prevent a re-formation of all-*trans* lycopene when reaching more neutral pH levels, possibly due to an association of *cis* lycopenes with biomolecules involved in the transport mechanisms. Further studies are needed to elucidate this hypothesis, including a simulation of the behavior of 13-*cis* lycopene in the intestines as well as a comparison of the intestinal absorption of 13-*cis* lycopene vs. all-*trans* lycopene.

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Evaluation of Anti-oxidant Activity Levels among Solanaceae Germplasm

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ABSTRACT

Improvement of tomato and pepper for antioxidant activity (AOA) could potentially benefit human health in many countries. We evaluated diverse entries of tomato (n=53) and pepper (n=46) for AOA and antioxidants (AO) for two years. Ample genetic variation was detected within both tomato and pepper to improve individual AO and AOA. Tomato entry means over years for inhibition of lipid peroxidation (ILP) ranged from 1.8–5.8 Trolox equivalent (TE) $\mu mol \cdot g^{-1}$ (fresh weight basis) with an overall mean of 3.97 TE $\mu mol \cdot g^{-1}$ and anti-radical power (ARP) values over years ranged from 2.5–6.8 TE $\mu mol \cdot g^{-1}$ with an overall mean of 4.11 TE $\mu mol \cdot g^{-1}$. Entry means for lycopene ranged from 0.04–23.09 $\mu mol \cdot 100g^{-1}$ with an overall mean of 7.12 $\mu g \cdot 100g^{-1}$. In general, means of the *L. pimpinellifolium* entries were significantly greater than *L. esculentum* for ARP, ILP, lycopene, vitamin C, phenolics, and soluble solids contents. Among the traits evaluated, total phenolics content showed the highest correlation with ARP (r=0.90**) and ILP (r=0.83**). Fruit size was negatively correlated with both ARP (r= -0.74**) and ILP (r= -0.71**). Capsicum entry means over years for ILP ranged from 13.0–61.8 TE $\mu mol \cdot g^{-1}$ (dry weight basis) with an overall mean of 27.4 TE $\mu mol \cdot g^{-1}$. Means of brown-fruited entries were significantly higher than the mean of red-fruited entries for capsanthin, zeaxanthin, cryptoxanthin, ß-carotene, α-tocopherol and γ-tocopherol. We found a significant positive correlations between ILP and vitamin C (r=0.86**) and ILP and total phenolics content (r=0.86**).

Key words: Carotenoids, Phenolics, Tomato, Pepper.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) and pepper (*Capsicum annuum*) comprising both pungent and sweet types, are among the most widely grown and consumed vegetables in the world. In terms of human health, tomato fruit and pepper fruit contain significant amounts of vitamins and antioxidant phytochemicals, including carotenoids, vitamins C and E, and phenolics. Carotenoids β-carotene and ß-cryptoxanthin both possess provitamin A activity and also function to help prevent and neutralize free radical chain reactions and ascorbic acid is an effective scavenger of superoxide, hydrogen peroxide, singlet oxygen and other free radicals (Clevidence *et al.*, 1997). Lycopene, the major carotenoid in tomato

fruit, is a powerful AO and has attracted much attention because of the linkage between lycopene-rich diets and lower risks of certain cancers, heart disease, and age-related diseases (Clinton, 1998).

Tomato and pepper genotypes are diverse in the types and concentrations of carotenoids (Stommel and Haynes, 1994; Wall *et. al.* 2001), levels of ascorbic acid, and phenolics (Abushita *et al.*, 1997). Selection for higher concentrations of particular AO is one strategy to improve tomato and pepper for overall antioxidant activity (AOA) (Tucker, 2003) Because AO in plants usually occur in mixtures, overall AOA may be greater or lesser than the sum of individual AO due to synergies or antagonisms (Arnao *et al.*, 2001; Halliwell *et al.*, 1995; Kochian and Gavin, 1999; Stahl *et al.*, 1998), or bioactivity of unknown AO (Arnao *et al.*, 2001). Because tomato and pepper are consumed daily in many countries, production and consumption of tomato and pepper varieties of higher AOA could potentially contribute to better human health worldwide. The objectives of this study were to assess genetic diversity within *L. esculentum* and *C. annuum* for AO and AOA.

MATERIALS AND METHODS

Entries included 53 tomato and 46 pepper representing diverse origins, fruit colors and sizes. Tomato and pepper trials were grown two years in fields of AVRDC-the World Vegetable Center from October 2001 – March 2002 (2002 season) and October 2002 – March 2003 (2003 season). Mean maximum and minimum temperatures during this period range from 22-30 °C and 12-22 °C. Average monthly rainfall is < 50 mm. Entries were replicated twice and arranged in a randomized complete block design. Plots included five plants in the 2002 season and ten plants in the 2003 season. Fully mature fruit were harvested from each plot once when most plants within plots had ripe fruit. Fruit were taken the same day to the laboratory samples were prepared for measurement of either fruit qualities, individual AO, or AOA. Samples for measurement of AO or AOA were stored in sealed bags at –70 °C until analysis time.

Entries were analyzed for AOA by anti-radical power (ARP) and/or inhibition of lipid peroxidation (ILP) methods as described in Hanson *et al.* (2004a) and Hanson *et al.* (2004b). ARP measures the capacity of different components to scavenge the ABTS radical cation as compared to the standard antioxidant Trolox (0 – 4 mM) in a dose response curve. Trolox is a vitamin E analog. The AOA of a sample for the ARP assay was measured within the linear relationship of concentration vs. optical density decrease, and presented as Trolox equivalent (TE) in μmol·g^{-1} fresh weight basis for tomato and TE μmol·g^{-1} dry weight basis for pepper. The ability of AO samples to inhibit lipid peroxidation at pH 7 was tested using linoleic acid as a substrate and AAPH as a free radical generator. The absorbance difference of the reaction without linoleic acid and with linoleic acid was represented as 100% of inhibition. Trolox (0 – 4 mM) was used as the antioxidant standard and AOA was expressed as TE in μmol·g^{-1} tomato fruit (fresh weight basis). Carotenoids, total ascorbic acid, and total soluble phenolics were determined as described in Hanson *et al.* (2004a) and Hanson *et al.* (2004b).

Variables were subjected to analysis of variance (ANOVA) over years with the SAS General Linear Model (GLM) procedure

(SAS Institute, Inc., Cary, N.C.) where year was regarded as a random effect, entry as a fixed effect, and the year x entry interaction mean square was used to test the significance of the entry mean square and linear contrasts.

RESULTS

Tomato

Tomato entry means over years in for ILP ranged from 1.8 – 5.8 TE μmol·g^{-1} and ARP values over years ranged from 2.5 – 6.8 TE μmol·g^{-1} (Table 1). The three *L. pimpinellifolium* accessions LA1582, LA1586 and LA1593 ranked first, second, and third highest, respectively for ARP, and second, eleventh and seventh, respectively for ILP. In general, small-fruited *L. esculentum* entries (<31g) produced significantly higher AOA than the medium and large-fruited entries. LA1315, LA1385, LA1455 and LA1456, all of which are small-fruited and classified as *L. esculentum* var. *cerasiforme* or South American landraces, demonstrated the highest levels of AOA among *L. esculentum* entries. All medium, large, and very large-fruited entries showed low or moderate AOA. For most entries, lycopene was the major carotenoid although the range of values was wide (0.04 to 23.09 mg·100 g^{-1} (fresh mass basis)). The three *L. pimpinellifolium* entries formed a distinct group of means that were significantly larger than all the *L. esculentum* entries. The *L. esculentum* entries producing high lycopene content included T5019 and T4065 containing *dg* + *og*^{c} and *hp* + *og*^{c} genes, respectively, as well as LA1315. CLN1314G and 97L63 containing the Beta gene showed low lycopene density and the highest β-carotene levels (3.73 and 3.38 mg·100 g^{-1}, respectively) compared to an average β-carotene content of 0.61 mg·100 g^{-1} for the red-fruited entries. Entry means over years for phenolics content ranged from 56-156 mg·100 g^{-1} with an overall mean of 90 mg·100 g^{-1}. Entry means over years for ascorbic acid content ranged from 12-40 mg·100 g^{-1}. Almost all *L. esculentum* entries of relatively high phenolics content were small-fruited and means of almost all medium or large-fruited entries fell below 90 mg·100 g^{-1}. Like phenolics content, entries producing the highest ascorbic acid content included the *L. pimpinellifolium* and small-fruited *L. esculentum* such as LA1455.

Pepper

ILP values ranged from 13.0-61.8 μmol·g^{-1} (DW basis) (Table 2). CAP178/77, the accession with the highest ILP value, also ranked first among entries for total phenolics content and second highest for ascorbic acid content. Over entries, capsanthin was the major carotenoid, accounting for about two-thirds of the total carotenoids. However, the range of entry means for capsanthin was vast (0-1585 μg·g^{-1}). ß-carotene entry means ranged from 2-292 μg·g^{-1}. The brown-fruited entries, in general, produced more carotenoids than red-fruited entries. Entries 'Guajillo Ancho' and 'Verdano Poblano TY' ranked first and second highest, respectively, for capsanthin content over years and ranked among the top five for the other carotenoids. Ascorbic acid values ranged from 2.3-21.0 mg·g^{-1}. Simonne *et al.* (1997) reported higher ascorbic acid content in brown bell peppers compared to other colored bell fruit and our results showed about 19% higher ascorbic acid content in brown- versus red- fruited accessions. Orange-fruited accessions, as a group, developed the highest ascorbic acid levels with about 48% greater than the mean

Table 1. Selected *Lycopersicon esculentum and L. pimpinellifolium* entries evaluated for antioxidant activity, antioxidants, and fruit qualities, AVRDC-the World Vegetable Center Taiwan, 2001-2002 and 2002-2003. Values are fresh weight means (± SE) averaged over years.

Entry name (carotenoid gene)	Species	Fruit size category	Antioxidant activity ILP	Antioxidant activity ARP	Antioxidants Lycopene	Antioxidants ß-carotene	Antioxidants Phenolics	Antioxidants Ascorbic acid	Fruit qualities Color	Fruit qualities Soluble solids
			TE (µmol•g^{-1})		(mg•100 g^{-1})				(a/b)	(%)
LA 1582	L.p.	very small	5.77 ± 0.07	6.84 ± 0.93	23.09 ± 0.67	0.88 ± 0.18	156 ± 22	39.7 ± 5.6	2.09 ± 0.19	7.7 ± 0.15
LA 1586	L.p.	very small	4.75 ± 0.79	6.47 ± 0.72	19.69 ± 1.10	0.89 ± 0.19	161 ± 20	36.6 ± 4.4	2.07 ± 0.05	8.6 ± 1.2
LA 1593	L.p.	very small	4.96 ± 0.53	6.40 ± 0.40	18.04 ± 0.71	0.89 ± 0.09	143 ± 12	28.5 ± 3.4	1.99 ± 0.02	7.1 ± 0.8
LA 1230	L.e.	very large	3.11 ± 0.40	3.00 ± 0.43	5.16 ± 0.56	0.46 ± 0.06	70 ± 3	15.2 ± 1.2	1.35 ± 0.10	4.2 ± 0.18
LA1247	L.e.	very small	4.49 ± 0.54	4.59 ± 0.40	8.93 ± 0.74	0.55 ± 0.09	96 ± 5	21.8 ± 3.7	1.62 ± 0.06	5.3 ± 0.13
LA1315	L.e.	very small	5.79 ± 0.23	6.26 ± 0.32	10.07 ± 1.89	0.66 ± 0.11	143 ± 11	26.5 ± 2.5	1.72 ± 0.16	6.3 ± 0.35
LA 1385	L.e.	small	5.50 ± 0.44	5.89 ± 0.65	8.23 ± 0.41	0.66 ± 0.05	130 ± 22	22.5 ± 3.3	1.63 ± 0.05	4.7 ± 0.41
LA 1455	L.e.	small	5.04 ± 0.44	5.79 ± 0.61	8.11 ± 1.32	1.00 ± 0.16	123 ± 22	28.6 ± 2.4	1.41 ± 0.10	5.0 ± 0.41
LA 1456	L.e.	very small	5.59 ± 0.43	6.08 ± 0.40	8.52 ± 1.35	1.15 ± 0.09	134 ± 14	26.2 ± 4.3	0.99 ± 0.02	4.4 ± 0.10
Nagcarlan	L.e.	very large	3.54 ± 0.33	3.75 ± 0.42	7.24 ± 1.66	0.70 ± 0.15	88 ± 5	20.8 ± 4.1	1.70 ± 0.17	4.7 ± 0.26
Walter	L.e.	very large	3.61 ± 0.37	2.96 ± 0.41	4.15 ± 0.47	0.36 ± 0.04	63 ± 4	12.1 ± 1.9	1.53 ± 0.13	4.7 ± 0.30
UC204A	L.e.	large	3.56 ± 0.45	2.80 ± 0.28	5.83 ± 0.95	0.42 ± 0.05	63 ± 1	12.3 ± 2.7	1.72 ± 0.15	3.7 ± 0.18
FMTT 33	L.e.	very large	3.55 ± 0.37	3.36 ± 0.34	5.09 ± 0.66	0.44 ± 0.07	72 ± 1	14.2 ± 2.5	1.62 ± 0.14	4.6 ± 0.09
T4065 (*hp*, *og*^{c})	L.e.	very large	2.42 ± 0.54	2.70 ± 0.28	10.64 ± 1.36	0.56 ± 0.03	63 ± 4	18.0 ± 1.7	2.16 ± 0.17	4.6 ± 0.06
T5019 (*hp*, *dg*)	L.e.	very large	3.40 ± 0.69	3.91 ± 0.37	9.30 ± 1.30	1.15 ± 0.14	87 ± 3	17.5 ± 2.6	1.81 ± 0.19	4.2 ± 0.21
CLN1314G (*B*)	L.e.	very large	2.86 ± 0.80	3.23 ± 0.32	1.18 ± 0.04	3.73 ± 0.28	68 ± 3	13.4 ± 2.6	0.73 ± 0.02	4.2 ± 0.14
97L63 (*B*)	L.e.	large	1.77 ± 1.09	2.53 ± 0.16	0.28 ± 0.08	3.38 ± 0.33	56 ± 4	12.9 ± 2.8	0.47 ± 0.03	4.2 ± 0.09
Waller-Duncan			1.36	1.16	2.68	0.29	29	5.3	0.27	1.0
Mean of all entries			3.97 ± 0.08	4.11 ± 0.09	7.21 ± 0.31	0.73 ± 0.05	90 ± 2	19.1 ± 0.6	1.55 ± 0.04	4.8 ± 0.07

Table 2. Capsicum accessions evaluated for antioxidant activity, antioxidants, and dry matter, AVRDC-the World Vegetable Center, Taiwan. Values are means calculated on a dry weight basis averaged over two years (± SE).

Entry	ILP	Cap-santhin	Zeaxanthin	Lutein	β-crypto-xanthin	β-carotene	Phenolics	Ascorbic acid	α-tocopherol	Dry matter
	TE (µmol•g^{-1})	(µg•g^{-1})					(mg•g^{-1})		(µg•g^{-1})	%
Perennial HDV	16.3 ± 3.6	462 ± 41	157 ± 27	52 ± 4	37 ± 4	80 ± 10	18.0 ± 1.6	6.5 ± 1.0	419 ± 16	29 ± 2
Nose Gay	21.1 ± 1.0	257 ± 17	28 ± 8	32 ± 3	12 ± 1	33 ± 5	19.7 ± 1.7	9.4 ± 1.3	57 ± 6	22 ± 1
P484/76	28.4 ± 2.7	0	0	133 ± 10	14 ± 4	61 ± 7	26.3 ± 2.1	9.4 ± 1.2	502 ± 98	10 ± 0.4
CAP178/77	61.8 ± 5.8	714 ± 61	155 ± 15	95 ± 8	81± 7	129 ± 17	37.6 ± 3.1	18.8 ± 1.9	551 ± 61	11 ± 0.3
P126/79	13.0 ± 0.7	398 ± 83	121 ± 14	58 ± 10	31 ± 3	41 ± 7	12.3 ± 0.8	2.6 ± 0.2	349 ± 31	36 ± 2
PI196181	20.4 ± 1.6	858 ± 172	114 ± 20	108 ± 23	73 ± 11	78 ± 4	19.5 ± 1.3	5.5 ± 0.5	507 ± 40	20 ± 0.2
PI124540	29.1 ± 1.8	749 ± 110	142 ± 30	90 ± 12	59 ± 11	90 ± 17	26.3 ± 2.3	12.8 ± 1.4	635 ± 44	20 ± 2
Mexico Serrano	24.2 ± 2.4	374 ± 55	47 ± 4	44 ± 6	24 ± 3	66 ± 11	22.5 ± 0.6	8.1 ± 2.1	306 ± 19	15 ± 0.3
Agronomico 9 Lines	53.0 ± 9.7	614 ± 168	136 ± 45	82 ± 24	65 ± 22	102 ± 31	32.4 ± 5.7	16.7 ± 3.6	423 ± 73	10 ± 0.3
Roque Mulato Poblano	32.1 ± 3.3	555 ± 123	264 ± 76	73 ± 16	58 ± 13	91 ± 12	28.2 ± 2.2	14.7 ± 1.1	466 ± 64	18 ± 1
Verdano Poblano TY	42.1 ± 2.8	1333 ± 349	207 ± 65	173 ± 50	103 ± 37	275 ± 70	32.6 ± 0.8	17.9 ± 1.1	859 ± 106	14 ± 0.2
Guajillo Ancho	38.0 ± 4.2	1585 ± 280	213 ± 47	210 ± 39	128 ± 38	292 ± 73	29.5 ± 3.8	14.8 ± 2.4	759 ± 181	20 ± 2
Prapadaeng	17.2 ± 0.8	570 ± 71	209 ± 43	70 ± 9	52 ± 4	56 ± 9	17.3 ± 1.6	7.2 ± 0.6	434 ± 18	25 ± 0.5
Lueng 4	24.3 ± 2.4	0	30 ± 23	189 ± 17	62 ± 13	111 ± 22	24.7 ± 1.8	10.6 ± 1.0	405 ± 15	19 ± 0.7
CAB	47.8 ± 1.6	416 ± 119	47 ± 11	53 ± 16	45 ± 10	84 ± 23	33.8 ± 1.3	17.1 ± 1.0	253 ± 29	10 ± 1
Waller-Duncan	6.7	442	97	63	42	96	4.4	2.9	206	4
Mean of all entries	27.3 ± 0.9	552 ± 30	115 ± 7	83 ± 4	53 ± 3	98 ± 6	23.5 ± 0.5	9.0 ± 0.4	455 ± 15	19 ± 0.5

of the red-fruited entries. Entry means over years for total phenolics ranged from 12.3-37.6 mg·g^{-1}. CAP178/77, Agronomico 9, Nacional AG-506, and CAB ranked among the top for both ascorbic acid and total phenolics. We found wide variation among entry means for α-tocopherol content (75-882 µg·g^{-1}) and Verdano Poblano and Guajillo Ancho were outstanding for high α-tocopherol content.

DISCUSSION

Genetic improvement of tomato for AOA and specific AO is possible given the large genetic variation present among tomato entries. However, entries outstanding for AOA and most AO in this study were exclusively *L. pimpinellifolium* or small-fruited *L. esculentum* and the medium or large-fruited entries in this study showed average or below average AOA. Although information is lacking on the improvement status of many entries, it is likely that the large-fruited entries were developed in plant breeding programs while *L. pimpinellifolium* and small-fruited *L. esculentum* are non-bred germplasm. Further work is required to determine if lower AOA of the larger, and presumably bred lines, is a consequence of increased fruit size or the result of selection against certain AO, especially phenolics, in the course of domestication/ plant breeding.

Among AO, total phenolics demonstrated the largest positive correlations with both ARP and ILP (r=0.90** and r=0.93**, respectively). Tomato breeders have given little attention to the phenolics content of tomato fruit but our study suggests that genetic improvement to increase total phenolics content is a worthy objective. All AO in this study, including total phenolics, were negatively correlated with fruit size (r= -0.72), suggesting that development of cultivars of commercial fruit size with high phenolics content may be difficult.

Ample genetic diversity exists within *C. annuum* for AOA and the AO evaluated in this study and genetic improvement of these traits is possible. *C. annuum* cultivar market types are highly segmented according to intended use, pod type (shape, size, and color), level of pungency, regional preferences and origins (DeWitt and Bosland, 1996). Hot pepper and sweet pepper constitute the major division of *C. annuum* (DeWitt and Bosland, 1996). Our data point to differences between pungent and non-pungent *C. annuum* in content of some AO although comparisons are complicated by the great difference in dry matter content between the two groups (22% versus 12% for pungent and non-pungent *C. annuum*, respectively). On a DW basis, non-pungent *C. annuum* as a group, contained higher concentrations of ascorbic acid (65%), total phenolics (36%), α-tocopherol (11%), and ß-cryptoxanthin (36%) compared to the group mean of the pungent accessions. Among the red-fruited entries, 'Verdano Poblano' and 'Guajillo Ancho', both 'Ancho' types originating from Mexico, ranked among the best entries for content of almost every AO, including individual carotenoids, ascorbic acid, α-tocopherol, and total phenolics. These two accessions are of particular interest and additional accessions of the Ancho/Poblano type should be investigated for their nutritional qualities.

Greater realization of the importance of phytochemicals in promotion of human health has prompted the broadening of dietary recommendations to include greater daily intake of a wider range of vegetables and fruit. This will be difficult in the tropics and subtropics where vegetable supplies are both highly seasonal and daily per capita availability fall drastically below the 200g recommended by AVRDC-the World Vegetable Center (Ali and Tsou, 1997). Increased phytochemical intake in the tropics requires multiple tactics, including production of tropically adapted cultivars with higher densities of micronutrients and AO; overcoming seasonality of vegetable production so affordable vegetables are available to poor consumers throughout the year; diversification of diets to include antioxidant-rich vegetable species.

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Antioxidant Capacities and Daily Antioxidant Intake from Vegetables Consumed in Taiwan

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ABSTRACT

Epidemiological studies have demonstrated protective effects on human health from consumption of vegetables and fruits. Information on plant-sourced antioxidant capacity and intake is crucial to better understand the health implications of various dietary patterns. The objective of this study was to determine daily intake of antioxidant activity (AOA) from vegetables for the Chinese population in Taiwan. AOA intakes from vegetables were calculated to be 2014 µmol TE/day for methanol-soluble antioxidants and 750 µmol TE/day for water-soluble antioxidants based on a vegetable consumption survey in Taiwan in 2000. Sweet potato leaves, leafy Chinese cabbage, bamboo shoot, and spinach contributed 46% of the daily AOA intake for methanol-extracted antioxidants; whereas, sweet potato leaves, spinach, white cabbage and radish contributed 57% of the water-soluble AOA intake. Highly consumed vegetables, including white cabbage, bamboo shoot and loofah, were low in AOA. Increased consumption of green leafy vegetables could greatly improve overall AOA intake.

Keywords: Antioxidant activity, Antioxidant intake, Vegetables.

INTRODUCTION

Increased vegetable consumption has been widely promoted in recent years because of their value to human diets by providing basic nutrients as well as non-nutrient phytochemicals associated with health maintenance and prevention of chronic diseases and cancers (Steinmetz & Potter, 1996). Dietary antioxidants are critical, especially when the human body is under oxidative stress due to imbalances between generation of reactive oxygen species and endogenous antioxidants (Launer & Kalmijn, 1998; Aruoma & Halliwell, 1997).

Numerous groups of phytochemicals in vegetables, such as carotenoids, ascorbic acid, α-tocopherol and polyphenols are recognized for their antioxidative ability (Salah *et al.*, 1995; Edge, McGarvey & Truscott, 1997; Papas, 2002). However, vegetables differ in the types and levels of antioxidants they contain. The synergies and

antagonisms of antioxidants in crude mixtures add complexity in attempts to explain their antioxidant capacities.

Instead of measuring antioxidant content, potential antioxidants can be evaluated for properties that inhibit generation of free radicals, directly scavenge free radicals, and delay or inhibit lipid peroxidation in vivo or in food. Several methods have been reported to characterize dietary antioxidants and determine their activities (Aruoma *et al.*, 1997). So far, no single method has been accepted to evaluate overall antioxidant capacity of food. A commonly used method, Trolox equivalent antioxidant capacity (TEAC, or ABTS method named in this study) reflects the ability of hydrogen- or electron-donating antioxidants to scavenge the ABTS radical cation ($ABTS^{\bullet+}$) compared with that of Trolox. This method is correlated with total phenolic content and antioxidant activity (AOA) measured by ORAC (oxygen radical absorbance capacity) and FRAP (ferric reducing antioxidant power) methods for common vegetables and fruits (Proteggente *et al.*, 2002; Pellegrini *et al.*, 2003). Other reported methods such as DPPH decomposition (Goupy *et al.*, 1999) and superoxide scavenging (SOS) (Murakami *et al.*, 1996) differ in the free radicals generated and their antioxidative mechanism. They have not yet been systematically compared.

The kinds and quantities of vegetables consumed by people vary greatly, depending upon culture, preference and availability. Per capita vegetable consumption in Taiwan is among the highest in the world. The antioxidant composition and capacity of vegetables relative to vegetables intake data are important to understand the health implications of various dietary patterns. The objective of this study was to assess the antioxidant capacities of common vegetables consumed by the Chinese population in Taiwan using three different assays (TEAC, DPPH and SOS) and to investigate the dietary antioxidant intake from vegetables based on collected data from a food consumption survey (Ali *et al.*, 2000).

MATERIALS AND METHODS

Chemicals

XOD (xanthine oxidase, grade III from butter milk, 1.0 unit/mg protein), NBT (nitro blue tetrazolium), ABTS (2,2-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid)), HRP (horseradish peroxidase, type VI-A, 1000 unit/mg solid), DPPH (1,1-diphenyl-2-picryl-hydrazyl) and linoleic acid were purchased from Sigma Chemical Co., St. Louis, MO. Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2- carboxylic acid) was purchased from Aldrich, St. Louis, MO. Other reagents used in this study were all analytical reagent grade.

Plant materials and preparations

The selection of the vegetables (Table 1) was based on household consumption survey conducted by Asian Vegetable Research and Development Center throughout Taiwan including 1457 households during 1998–1999 (Ali *et al.*, 2000). It was developed on the basis of 24 hrs recall.

Fresh materials were purchased in 2001 – 2003 from local markets in southern Taiwan. About 2 kg of edible portion of each fresh sample were collected and washed with tap water followed by distilled water, cut into 1 x 1 cm pieces and well mixed. Exactly 20 g of cut samples were homogenized with 80 mL of deionized water and 20 g for 80 mL of methanol, respectively, in a homogenizer. Juices of water extracts were squeezed from samples with a steel press. Both water and methanol extracts were centrifuged at 15,000 rpm for 10 min at 10℃, divided into several 1.5 mL volume in microtubes, and frozen at –70 ℃ immediately for subsequent AOA analysis. Each AOA value was an average of two replications per extract. About 200 g of chopped vegetables were hot-air dried (50℃ for 2 days), ground into fine powder for dry matter determination (135 °C/ 4 hr).

Antioxidant activity assay (TEAC method)

The $ABTS/H_2O_2/HRP$ decoloration method was carried out as described in Arnao *et al.* (2001) with some modifications indicated in Hanson et al. (2004). The capacity of different components to scavenge the ABTS radical cation was compared to Trolox (0, 1, 2, 3 and 4 mM) in a dose response curve. The reaction mixture was prepared freshly containing 20 mM ABTS, 0.41 mM H_2O_2 and 50 units of HRP in 50 mM Na-phosphate buffer (pH7.5) in a total volume of 10 mL. The reaction mixture was further diluted to 100 mL with 50 mM phosphate buffer for water-extracted samples or diluted to 100 mL with ethanol for methanol-extracted samples. Stable absorbance at 730 nm was obtained in 2 min and reached OD_{730nm} = 1.0. Then 20 µL of the plant extract with appropriate dilution in water or methanol was added to the 1 mL of the reaction medium. The decrease in absorbance, which is proportional to the ABTS radical quenched, was determined after 5 min.

DPPH method

The DPPH assay was performed as described in Goupy *et al.* (1999) with some modifications. The radical form of DPPH has an absorbance at 517 nm, which disappears upon reduction by H • pulled from antioxidant compounds. Each plant extract was diluted with water (or methanol) to an appropriate concentration in a total volume of 2 mL; then 2 mL of 60 µM DPPH in water (or methanol) was added. The absorbance decrease was determined after 30 min in the dark. Trolox (0, 4, 8, 12, 16, 20 µM) was used as standard.

SOS method

The SOS was measured by the xanthine/XOD system as described by Murakami et al. (1996) with some modifications. The superoxide radical is generated from the oxidation of xanthine to uric acid by XOD. The radical reacts with NBT to cause a color change from light yellow to dark purple, which can be measured at $OD_{560\ nm}$. The ability of sample antioxidants to scavenge superoxide radicals can be determined by inhibition of NBT reductions. Methanol extracts of samples were N_2 air dried and dissolved in water with a few drops of Tween 20 before the assay. In a 20 mL tube, we added 20 µL of sample, 500 µL of PBS (pH7.2) containing 0.24 mM NBT and 0.4 mM xanthine, followed by 500 µL of a XOD solution (0.049 unit/mL) in PBS. After incubation at 37 °C for exactly 20 min, the reaction was stopped by adding 1 mL of a sodium dodecyl sulfate solution (69 mM). Visible absorption of the reaction mixture at 560 nm was measured. The absorbance difference of the reaction without XOD and with XOD was represented as 100% of NBT reduction inhibition. A sample blank was included without NBT to determine the sample's color interference to readings. A sequence of sample dilutions (at least 4

dilutions) with water was carried out until the NBT inhibition fell below 10%. Ascorbate (0, 0.5, 1, 2, 5, 10 mM) was used as standard.

RESULTS AND DISCUSSION

The 27 most consumed vegetables in Taiwan were identified by a food consumption survey and their AOA of water and methanol extracts were evaluated using three AOA assays. So far there is no optimal solvent to extract all antioxidants from plants. Vegetables are commonly prepared and consumed in a water-rich food matrix; methanol is a widely used solvent to extract phytochemicals in plants, especially for Chinese herbs, and ethanol with similar polarity to methanol is commonly added in Chinese household preparation by using rice wine containing about 18% ethanol. Thus we used water and methanol as the solvents in this study to reflect the AOA in food matrix instead of using combinations of solvents with various polarities in acidic or alkaline condition. The hydropholic AOAs were not included in this study due to their very low values compared to water and methanol extracts in our preliminary test. The low contribution of hydrophobic antioxidants to overall AOA was also reported by Wu et al. (2004).

AOA of the most consumed vegetables

The AOA values of vegetables for the six assays and overall AOA ranking are listed in Table 1. he overall rank was average rank over all assays. The ranking of vegetables for each assay, though different, showed similar trends. Vegetables ranked in the top five in one or more AOA assays included sweet potato leaf, old ginger, amaranth, spinach, eggplant, pak choi, leafy Chinese cabbage, tomato, welsh onion and kang-kong. Among these, sweet potato leaf and ginger were in the top five vegetable lists in every assay. Sweet potato leaf showed the greatest AOA by ABTS and DPPH methods, whereas ginger was the vegetable with highest SOS activity. Most green leafy vegetables exhibited high AOA by ABTS and DPPH methods, where 5 of the top 7 by overall ranking were green leafy types. It is noteworthy that eggplant, tomato and welsh onion had high SOS activity but relatively low AOAs by ABTS and DPPH methods. The lowest-ranking vegetables for AOA were wax gourd, loofah, cucumber and bottle gourd.

Regarding the extraction solvents, methanol-soluble antioxidants exhibited greater AOA by ABTS method for the vegetables tested; incontrast, the water extracts of 18 vegetables out of 27 showed higher AOA values by DPPH method. For, SOS, 17 vegetables showed greater activity with water extracts versus methanol extracts. However, vegetables with DPPHw > DPPHm are not necessary the same as those with SOSw > SOSm. The greatest AOA values by each method were all found from methanol extracts of vegetables.

Correlation coefficient between AOA values determined from six assays were all significant. The highest correlation coefficient was obtained with AOA values by ABTSm and DPPHm ($r=0.9548$, $p<0.0001$), whereas AOA values by SOSm and DPPHw ($r=0.3955$, $p=0.0412$) and AOA values by SOSm and DPPHw ($r=0.4436$, $p=0.0205$) had smaller but statistically significant correlation coefficient.

Table 1. Antioxidant activity[1] (AOA) of common vegetables[2] in Taiwan and overall AOA rank[3].

Scientific name	Common name	AOA Rank	ABTSm TE	ABTSw TE	DPPHw TE	DPPHm TE	SOSw AE	SOSm AE
Brassica oleracea	Cabbage, white	20	1.60	0.80	1.45	0.70	0.3	ND
Bambusoideae spp	Bamboo shoot	17	3.28	0.52	0.39	0.20	8.5	5.9
Luffa cylindrica	Loofah	26	0.86	0.25	0.12	0.29	ND	ND
Raphanus sativus	Radish	15	3.21	3.50	2.42	0.38	6.6	ND
Lactuca sativa	Heading lettuce	23	1.75	0.50	0.90	0.20	0.0	ND
Ipomoea aquatica	Kang kong	14	4.75	0.35	0.94	0.53	18.2	11.3
Ipomoea batatas	Sweet potato, green leaf	1	28.33	15.49	12.12	19.40	24.7	118.9
Brassica campestris	Leafy Chinese cabbage	7	10.71	3.94	3.03	3.56	6.8	9.2
Spinacia oleracea	Spinach	4	10.30	5.10	7.90	5.25	31.9	ND
Brassica campestris	Chinese cabbage	19	2.40	1.05	1.25	0.65	0.0	ND
Daucus carota	Carrot	18	2.85	0.31	0.51	0.41	9.2	5.8
Cucumis sativus	Cucumber	25	1.40	0.24	0.21	0.14	1.7	ND
Allium fistulosum	Welsh onion	13	6.15	1.64	1.47	0.43	6.8	13.2
Lagenaria siceraria	Bottle gourd	24	1.62	0.51	0.20	0.10	3.7	ND
Brassica oleracea	Cauliflower	8	7.57	2.11	2.59	2.52	17.7	4.6
Benincasa hispida	Wax gourd	27	0.21	0.18	0.58	0.03	ND	ND
Lycopersicon esculentum	Tomato	10	3.41	3.20	0.98	0.81	13.0	28.0
Apium graveolens	Celery	22	2.05	0.45	0.65	0.35	4.0	0.8
Momordica charantia	Bitter gourd	9	7.80	1.92	1.62	3.65	9.7	4.0
Amaranthus spp	Amaranth, green	3	14.07	3.58	4.02	10.35	16.6	9.8
Vigna unguiculata	Yard long bean	11	9.10	1.66	1.57	3.81	8.0	3.7
Phaseolus vulgaris	Kidney bean	12	10.30	1.50	1.60	4.40	5.7	ND
Zingiber officinale	Ginger, old	2	15.31	11.04	6.40	6.16	32.4	131.0
Solanum melongena	Eggplant	5	7.12	3.77	1.29	4.15	18.9	62.5
Zizania latifolia	Water bamboo	21	2.22	0.82	0.76	0.41	4.9	ND
Vigna radiata	Mungbean sprouts	16	8.41	1.12	1.27	0.54	5.8	0.8
Brassica campestris	Pak Choi	6	10.84	3.17	3.52	4.62	12.9	0.8
	Mean		6.58	2.55	2.21	2.74	9.9	15.2
	SD		6.08	3.45	2.70	4.18	9.2	34.2
	Min		0.21	0.18	0.12	0.03	ND	ND
	Max		28.33	15.49	12.12	19.40	32.4	131.0

[1] Values are means, n=2, in μmol TE/g or μmol AE/g on fresh weight basis. TE, Trolox equivalent; AE, ascorbate equivalent. ND= not detected.

[2] The selection of vegetables was based on the food consumption survey in Taiwan (Ali et al. 2000). Vegetables are in the sequence of the daily consumption from high to low.

[3] The overall ranking was based on average rank over each AOA assay.

AOA Intake from Vegetables

In Table 2, the average antioxidant per capita intake from vegetables on the basis of AOA values by ABTS method were calculated to be 2014 µmol TE/day for methanolic antioxidants and 750 µmol TE/day for aqueous antioxidants. Sweet potato leaves, leafy Chinese cabbage, bamboo shoot, and spinach together contributed the 46% of daily intake of methanol-soluble antioxidants; whereas, sweet potato leaves, spinach, white cabbage and radish contributed 57% of AOA from water-soluble antioxidants. In spite of high AOA of amaranth and ginger, their contribution to daily AOA intake was only about 4 – 5 % of the total AOA due to their low consumption. White cabbage, bamboo shoot and loofah were the most consumed vegeables, however, their overall AOA rankings were 20, 17 and 26, respectively, and their AOA contribution to daily antioxidant intake was only 1 – 6%.

Table 2. Daily AOA intakes[1] from vegetables in Taiwan.

	Daily intake (g/person)	Daily antioxidant intake[2]			
		Methanol extractable AOA		Water extractable AOA	
		µmol TE	%	µmol TE	%
Cabbage, white	45.5	73	4%	66	9%
Bamboo shoot	34.8	114	6%	14	2%
Loofah	25.0	22	1%	3	0%
Radish	22.2	71	4%	54	7%
Heading lettuce	21.3	37	2%	19	3%
Kangkong	20.3	96	5%	19	3%
Sweet potato, green leaf	18.6	526	26%	225	30%
Leafy Chinese cabbage	15.4	165	8%	47	6%
Spinach	10.9	112	6%	86	11%
Chinese cabbage	10.8	26	1%	14	2%
Carrot	9.9	28	1%	5	1%
Cucumber	9.4	13	1%	2	0%
Welsh onion	9.0	55	3%	13	2%
Bottle gourd	8.9	14	1%	2	0%
Cauliflower	8.6	65	3%	22	3%
Wax gourd	8.4	2	0%	5	1%
Tomato	7.9	27	1%	8	1%
Celery	7.8	16	1%	5	1%
Bitter gourd	7.7	60	3%	12	2%
Amaranth, green	7.0	99	5%	28	4%
Yardlong bean	6.9	63	3%	11	1%
Kidney bean	6.4	66	3%	10	1%
Ginger, old	6.4	97	5%	41	5%
Eggplant	6.3	45	2%	8	1%
Water bamboo	5.9	13	1%	4	1%
Mungbean sprouts	5.7	48	2%	7	1%
Pak Choi	5.4	59	3%	19	3%
Total	352	2014	100%	750	100%

[1] The data source of the consumption amounts was referred to Ali *et al.* (2000).

[2] The AOA intakes were calculated based on AOA values by ABTS method.

The types of vegetables eaten daily were very different in the US versus Taiwan. Antioxidants from dry beans and peas contributed 64% of the daily AOA in the US, followed by lettuce (8%) and tomato (8%). The major antioxidants were quercetin and kaempferol flavonoids in beans and peas (Plumb *et al.*, 1999), quercetin in lettuce and carotenoids in tomato (Bravo, 1998). In Taiwan, antioxidants from leafy vegetables contributed the most to total AOA, especially sweet potato leaves, leafy Chinese cabbage, spinach, kangkong and amaranth. Their major antioxidants were beta-carotene, lutein, vitamins C, E, phenolic acids (such as gallic acid, chlorogenic acid, ferulic acid) and flavonols (such as quercetin and kaempferol) (Bravo, 1998) It seems that Taiwan people consume both more amount and types of antioxidants. The health benefits of antioxidants derived from different types of vegetable might be different and worth further investigation.

It is worth noting that sweet potato leaf contributed almost one-third of overall AOA intakes from vegetables. Sweet potato leaves are eaten as a cooked leafy vegetable in many countries (Villareal *et al.*, 1982) and the leaves contain anthocyanins and phenolic acids (Islam *et al.*, 2002a, 2002b), and showed anti-mutagenic properties (Yoshimoto *et al.*, 2002).

Vegetable consumption in Taiwan is one of the highest in the world with a daily per capita intake of about 350–420 g including a diversity of species (Ali et al. 2000; Wu et al. 1999). Several species including sweet potato leaves, amaranth and ginger have higher AOA; however, the most consumed vegetables tended to have lower AOA. To increase antioxidant consumption in Taiwan, people should eat more green leafy vegetables or additional vegetables with high AOA. In our recent survey of more than 120 vegetables species for AOA We found several very high AOA plant species, such as Chinese cedar and Moringa. Given the wide range of AOA among edible plants, there is great potential to increase antioxidant consumption. Because AOA of certain species such as Chinese cedar is 100 −1000 times greater than common vegetables, even consumption of a small quantity could greatly increase AOA consumption.

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Anthurium Shipping and Cold Tolerance

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ABSTRACT

Postharvest handlers and shippers of tropical ornamentals are limited by their products chilling susceptibility, short vase life, long distances to major markets, and the need for insect disinfestation. Frequently tropical ornamentals shipped to temperate areas during the winter are exposed to freezing temperatures during transit and are severely damaged. Packages can provide some protection. Cartons with ½" thick Styrofoam on all sides gave protection for up to 5 hours and could possibly give protection up to 8 hours exposure to -15℃. After freezing exposure, the cartons should be immediately unpacked to avoid longer exposures to low temperatures. The shredded newspaper traditionally used for packing in Hawaii should not be wet to avoid freezing when in contact with the flowers. Anthurium cultivars varied in their tolerance to low temperature exposure with greater tolerance being shown by Marian Seefurth, Lavender Lady, Lady Ann, Tropical Ice and Mickey Mouse.

Key words: Anthurium, Shipping, Cold Tolerance.

INTRODUCTION

The anthurium (*Anthurium andraeanum*) flower consists of a modified leaf; the glossy spathe, and the upright flower spadix in the middle of the spathe with more than 300 spirally attached minute flowers (Watson and Shirakawa, 1967). Cultivars have a wide range of colors and forms and the vase life can seasonally vary from 8 to 69 days (Paull et al., 1992). Conflicting evidence exists as to whether bacterial or physiological blockage of the vascular tissue reduces postharvest life. The end of the vase life is shown as loss of spathe glossiness and then spathe blueing due in part to water loss. The majority of the water loss occurs from the spadix.

All of Hawaii's ornamentals are shipped by air, while most foliage plants and nursery stock are sent by sea (Akamine, 1976; Paull, 1991; Criley *et al.*, 1993). The air-shipped product sent to major mainland markets during winter frequently are exposed to freezing temperature. The freezing temperatures are most commonly experienced during movement from the destination airport to the retail outlet. Packages, suitable to protect the ornamentals during cold exposures have been developed. Most of these packages use Styrofoam sheeting and reflective films. Similar packages can be used to protect products during exposure to heat during the summer.

Our recent research has focused on the evaluation of different packages to protect anthuriums during shipment. The second aspect has been to determine the relative sensitivity of anthurium cultivars to cold exposure. Before presenting the results of this research, I would like to review anthurium postharvest handling to provide a context for handling decisions.

POSTHARVEST HANDLING

Maturity of anthurium flowers is determined by the proportion of open flowers on the spadix. In immature anthuriums, the spadix is smooth. Flower opening starts at the base of the spadix and proceeds upwards; spadices with open flowers are noticeably rough. Although producers in some countries harvest anthuriums when as little as 20% of the spadix is rough, Hawaiian growers harvest flowers when only 25% of the spadix is still smooth (75% of the flowers have opened). Harvesting anthuriums when more mature increases overall vase-life. For maximum vase life, flowers should be harvested when the spadix is 50 to 75% rough.

Waxing and silver nitrate treatments increase vase life (Paull and Goo, 1982). However, waxing can cause spathe blueing of some cultivars and impart an undesirable gloss. Silver nitrate runs into the problems of cost and disposal of spent solution. The vase life can also be increased by benzyladenine applied as a dip or as a spray (Paull and Chantrachit. 2001). Different anthurium cultivars do show differences in vase life responsiveness to BA treatment from a 20% reduction to a 2.5 fold increase in vase life. The maximum vase life increase is obtained at about 100 mg L^{-1} BA for 'Leilani', 'Blush Oishi', and 'Purple Arcs'. 'Marian Seefurth' reached the maximum vase life at 150 mg L^{-1} and gave only a slight further increase in vase life at 200 mg L^{-1}. 'Ozaki' showed a slight increase in vase life at 100 mg L^{-1} (Figure 1), while 10 mg L^{-1} as used by Shirakawa et al., (1964) gave little or no increase in vase life. There was no reduction in vase life at higher BA concentrations.

The storage recommendation is between 12.5 to 20℃ (Paull, 1987). Temperatures less than 10℃ leads to chilling injury for which the symptoms are purpling and browning. Anthurium flowers can be stored for at least 1 week if packed in moist shredded newsprint and held at 15 ℃. However, detailed information on exposure temperatures and duration are unavailable.

ANTHURIUM COLD TOLERANCE

Blueing of anthurium spathes and the browning or water soaking of tissue were the symptoms most commonly observed immediately after freezing exposure. Sometimes, symptoms were not evenly distributed. It is suspected that these uneven injuries were caused by the direct contact of the spathe with the wet, frozen shredded paper used for packing. One day after unpacking, the symptoms of injury seen were discoloration and browning of the tissue. Most of these symptoms usually occurred at the tip and edge of the spathe and spadix. Marian Seefurth, Lavender Lady, Lady Ann, Tropical Ice and Mickey Mouse are anthurium varieties that are more tolerant to cold temperature exposures (Table 1). Under the same condition Kalapana Obake, Lehua red and Midori green showed marked injury.

PACKAGING TO AVOID COLD INJURY

We compared the ability of seven

different box systems to the standard ornamental's shipping box to prevent damage to flowers from freezing exposure. About 24 hours after packing, the boxed were exposed to -10°C to -15℃ for up to 5 hours, then returned to ambient temperatures (22℃) unpacked, evaluated, and vase-life determined. The different boxes evaluated were: #1.Control - Gift box, plastic liner, shredded paper, and sealed, then held at room temperature. #2. Control - Gift box, plastic liner, shredded paper, and sealed and exposed at -16℃. #3. Double box system - gift box, 4 sheets of newspaper, 2 sheets of 1/16th foam, shredded paper, 8 sheets newspaper used for #12 box/double box and seal. #4. Foam board - gift box, ½" foam board all sides (6 pieces), plastic liner, shredded paper. #5. 1/8" Foam sheet - gift box, 1 sheet 1/8" foam sheet on bottom, 1 sheet 1/8" foam liner, closed liner, 1 sheet 1/8" foam on top and seal. #6. Holographic box- plastic liner, shredded paper, and sealed. #7. Holographic box-double box system #12 box, 4 sheets newspaper, 2 sheets 1/16th foam, shredded paper, 8 sheets newspaper used for #12 box/double box and sealed. #8. Holographic box (foam board) - ½" foam board, plastic liner and seal. #9. Holographic box - 1 sheet 1/8" foam sheet on bottom, 1 sheet 1/8" foam liner, and close liner, 1 sheet 1/8" foam on top and sealed.

Boxes #4 and #8 that have a ½" foam sheets gave the best insulation protection. These two boxes were able to maintain the temperature above 5°C in the box, even when they were exposed to -15°C for 5 hours (Fig. 1). The carton with only one 1/8" foam sheet (#9), was the third best in insulating ability. The minium temperature inside the box was 2.5℃. Treatment #2 and #6 cartons did not prevent the air temperature inside the box from dropping below freezing. The double box system, (# 3 and #7) showed a similar temperature profile to treatment #5. The temperature inside these boxes dropped to below zero after five hours' cold exposure.

Table 1. The shelf life of anthurium varieties exposed to -15℃ for 5 hours held in three different cartons: #1.Control - Gift box B, plastic liner, shredded paper, and sealed, then held at room temperature. #2. Control - Gift box B, plastic liner, shredded paper, and sealed and exposed at -16℃. and #8. Holographic box (foam board) - ½" foam board, plastic liner and seal.

	Injury from freezing exposure			Vase Life (days) & Box #		
	Severe	Moderate	None	#1	#2	#8
Leilani Red	j			18	1 *	18
Lehua Red		j		21	0 *	1
Marian Seefurth Pink		j		21	4	6
Midori Green	j			35	0 *	0 *
Kalapana Obake	j			13	0 *	0 *
Tropical Fire	j			8	1 *	1 *
Lavender Lady			j	11	15	17
Lady Anne white		j		8	0 *	6
Tropical Ice	j			11	0 *	8
Mickey Mouse		j		27	0 *	4
Anthurium Leaf	j			11	0 *	11

* Injury present upon removal from freezing exposure.

The injury incidence was significantly reduced in the box packed with newspaper when compared with the box which showed similar temperature but without newspaper, when compared with carton #4 and #8. Dry newsprint in carton #4 served as an added insulator for cold temperature. The flowers in treatment cartons #3, #4 and #7 showed the least chilling injury while those in cartons #2, #6 and #9 showed the most damage. It was surprising to find that the anthurium in carton #9 showed substantial injury, though the temperature profile indicated it had a relatively higher temperature inside. The injuries may be due to the direct contact of anthurium spathe with cold air or wet newspaper surfaces.

SUMMARY

Marian Seefurth, Lavender Lady, Lady Ann, Tropical Ice and Mickey Mouse are anthurium varieties that are more tolerance to cold temperature. Under the same condition, Kalapana Obake, Lehua red and Midori green showed marked injury. Cartons with ½" thick Styrofoam on all sides gave protection for up to 5 hours and could possibly give protection up to 8 hours. After low temperature exposure, the cartons should be immediately unpacked to avoid longer exposures to low temperatures.

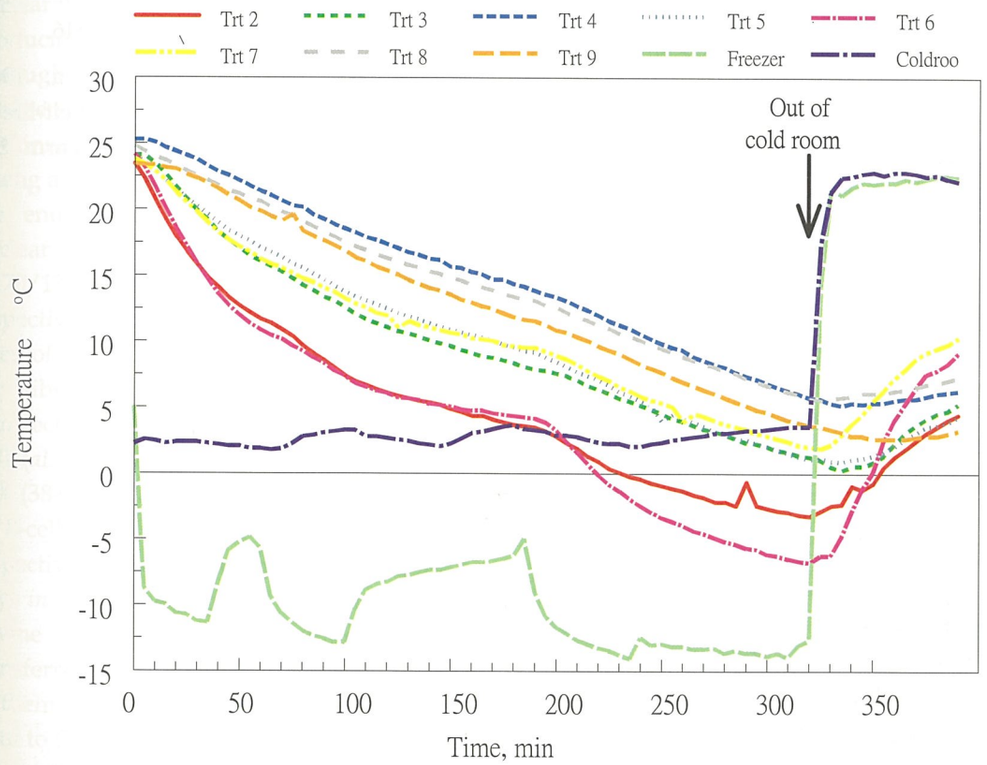

Fig. 1. Temperature profiles inside various packed flower boxes exposed to as low as -15℃.

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Studies on Nuclear Transfer Techniques for Generation of Transgenic-cloned Calves and Goat

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ABSTRACT

The purpose of this study was to evaluate the feasibility of using somatic cell nuclear transfer (SCNT) technique for producing the clone transgenic bovine. Through the international cooperation between Professors Jerry Yang at University of Connecticut (UCONN) and Winston T. K. Cheng at National Taiwan University (NTU), the enucleation rate and fusion rate of nuclear transferred (NT) embryos were 89% (1170/1311) and 88% (938/1064), respectively. Moreover, the development rates of the NT-embryos cloned from adult ear fibroblast cells of which had been transfected with human blood clotting factor VIII (*aLA-hFVIII*) gene were 90% (670/741), 59% (384/741), and 38% (278/741) reached 2- ~ 8-cells, 16-cells and blastocyst stage, respectively, after having been cultured for 7 days *in vitro*. One hundred and ninety-three bovine cloned transgenic embryos were transferred into 82 recipient females. Eleven of them were pregnant, with only 5 giving birth to 5 cloned calves. Unfortunately, one of the five cloned calves was dead after birth. While none of those four survived cloned calves was evidenced harboring with the *aLA-hFVIII* transgene when their genomic DNA had been subjected to the southern blot analysis, respectively, one cloned dairy goats carrying the *aLA-hFVIII* transgene has been successfully generated based on the same strategy described above. In conclusion, this study has successfully produced five cloned calves and one cloned-transgenic goat by SCNT technique.

Key words: Bovine, Nuclear transfer, Fibroblast cell, Transgenic, *Human blood clotting factor VIII (aLA-hFVIII)*.

INTRODUCTION

Somatic cell nuclear transfer (SCNT) method has been used to clone several mammalian species including sheep (Wilmut *et al.*, 1997), cattle (Kato *et al.*, 1998), goats (Baguisi *et al.*, 1999), pigs (Polejaeva *et al.*, 2000), rabbits (Chesne *et al.*, 2002), mice (Wakayama *et al.*, 1999), horses (Galli *et al.*, 2003), mules (Woods *et al.*, 2003), and cats (Shin *et al.*, 2002). Several adult somatic cell types, including mammary gland cells (Wilmut *et al.*, 1997), oviduct epithelial cells (Kato *et al.*, 1998), cumulus cells (Wakayama *et al.*, 1998), mural granulosa cells (Wells *et al.*,

1999), muscle cells (Shiga *et al.*, 1999), uterine epithelial cells (Cho *et al.*, 2000), and ear fibroblasts (Kubota *et al.*, 2000) were used to produce these clones.

In Taiwan, both transgenic (Tg) pigs and Tg dairy goats harboring human clotting factor IX and factor VIII cDNA(s) have been generated by direct microinjection of foreign DNA into the pronucleus of zygotes, respectively, the overall efficiency of this process is only around 1%. The technique of SCNT in mammals has been developed to produce cloned and transgenic animals. Moreover, the technique of SCNT has been proven more successful in generating transgenic animals than that pronuclear microinjection (Schnieke *et al.*, 1997). The advantage of using NT to produce transgenic animals is the ability to use preselected genetically modified cells as donor nuclei (Schnieke *et al.*, 1997).

Therefore, in this study, we evaluated the feasibility of using SCNT technique to produced cloned-transgenic livestock. We have successfully produced five cloned calves and one cloned-transgenic goat so far.

MATERIALS AND METHODS

1. Oocyte collection and enucleation

(1) Oocytes collection in goat

In vivo matured caprine oocytes were used for nuclear transfer. Estrus of donor does were synchronized by intravaginal releasing device with 366 mg of progesterone (CIDR®, AHI Inc. New Zealand). Each animal was injected with 125 μg of the $PGF_2\alpha$ analogue (cloprostenol, Estrumate®, Coopers, Germany) on day 11. For superovulation, animals were injected with a total of 18 units of pFSH (Tenka Pharmacy Co. Japan) plus a total of 11.2 units of LH (Sigma L-5269), which were divided into 6 injections for three days 24 h before removal of the CIDR. Single dose of 1500 IU of hCG (Gona-500®, China Chemical & Pharmacy Co. Taiwan) was administered 8 h after the last FSH injection. The surgery of the oocyte donors was performed 22 h after hCG administration. The cumulus-oocyte complexes (COCs) were recovered by flushing the uterine horns with Dubelco's phosphate-buffered solution (PBS) using an 18G plastic catheter. Collection of the COCs was performed from the cranial part of the uterine horn based on the protocol described by Shen (2003). The COCs had their cumulus cells removed by gentle pipetting in the hyluronidase (Sigma H-3506) solution (1 mg/ml in PBS) for 3 min. Only the oocytes with first polar body (PB1) were used for enucleation.

(2) Oocytes collection in cattle

The cattle ovaries were taken from slaughterhouse. After ovaries collected, the COCs were collected from follicles (2-8 mm diameter) on the surfaces of the ovaries by a 1 ml syringe with No. 18 needle under negative pressure. The quality well and cumulus cells completed oocytes were chosen for *in vitro* maturation. According to the bovine oocyte maturation step described by Shen (2003), ten to twenty of the COCs were transferred in the maturation medium (5% FCS M199) and cultured in the 38.5°C, 2% CO_2, 98% air and saturated humidity condition for 18-19 hours. The externally matured oocytes containing the first polar body were chosen for enucleation.

(3) Enucleation of goat and cattle oocytes

The enucleation of goat and cattle oocytes were achieved by cutting the zona pellucida and pushing out the PB1 and its surrounding cytoplasm with a glass needle. Successful enucleation was confirmed by

Hoechst 33342 staining of the remaining ooplasm (Kubota *et al.*, 1998).

2. Collection and culture of ear fibroblast cells

Skin biopsies were obtained from the ears of a 2-year-old Alpine doe and a 2-year-old Holstein cow of which with high milk yield. The ear tissues were cut into small pieces (3 mm^2) and then cultured as tissue explants in cell culture medium at 38.5 ℃ in a humidified incubator containing 5% CO_2 in air. When fibroblast cells had formed monolayers surrounding the tissue explants after 10 days culturing, the explants were then removed and the fibroblast cells cultured to confluency. The cell strain was routinely maintained on dishes until passage 10 and then stored frozen (Kubota *et al.*, 2000).

3. Transfection of foreign gene into ear muscle fibroblast cells:

A plasmid contained the full-length cDNA sequences of human coagulation factor Ⅷ (hF Ⅷ) and the neomycin resistance gene driven by the bovine α-lactabumin (αLA) promoter and the simian virus-40 (SV-40) promoter, respectively, was linearized and delivered into the ear skin fibroblast cells using electroporation method (150 V, 10 msec, 2 pulses). The transfected cells were suspended in the medium contained DMEM supplemented with 10% FBS and subjected to *in vitro* culture at 37 ℃ in a humidified atmosphere of 5% CO_2. Positive cells harboring the transgene(s) were selected for a period of 20 days using this same medium containing 1 mg/mL neomycin as the method described by Schnieke *et al.* (1997). Selected cell lines were frozen into small aliquots for later use in nuclear transfer studies.

4. Preparation of donor cells

Donor cells harboring the αLA-hF Ⅷ gene, after thawing and plating into 4-well plates and culture in DMEM plus 10% FBS and 1% penicillin/streptomycin till confluent, had been subjected to serum starvation (0.5% FBS in DMEM) for 5~8 days before they were collected by trypsinization, and then resuspended in DMEM containing 0.5% FBS for nuclear transfer (Kubota *et al.*, 2000). Both enucleation of reciepient oocytes and transfer of donor cells were manipulated within a drop of the handling medium (PBS containing 10% FBS). For nuclear transfer, each of the donor cells, sized less then 20 μm with smooth plasma membrane, was picked up with a manipulation pipette and slipped into perivitelline space of the enucleated oocyte. Immediately after cell transfer, cell-cytoplast couplets were subjected to electrical fusion within mannitol fusion medium (0.32 M mannitol + 100 μM $CaCl_2$ + 100 μM $MgSO_4$ + 0.01 mg/ml BSA (sigma A-8806)) according the procedures described by Kubota *et al.* (1998). Briefly, each of the manipulated embryo was aligned by a pair of electrodes so that the contact surface between the donor cell and the enucleated oocyte was parallel to the electrode surface and a single pulse with 10 μsec in pulse length at 1.34 kV/cm electrical field strength was given by using the BTX Electrocell Manipulator 2001. After exposure to the fusion pulse, the fused couplets were placed and incubated in drop of 80 μl TCM-199 plus 5% FBS and 1 μl/10 ml gentamycin under mineral oil incubated at 38.5℃ in 2% CO_2 till activation (Shen *et al.*, 2003).

5. Cell insertion and electrofusion

The enucleated oocytes and dispersed donor cells were manipulated in PBS containing 10% FBS. The donor cell (<20 μ

m) with smooth plasma membrane was picked up with a manipulation pipette and slipped into the perivitelline space of the enucleated oocyte. Before fusion, a cell-cytoplast couplet was aligned and gently pressed by a pair of electrodes in the fusion medium [0.32 M mannitol + 100 μM $CaCl_2$ + 100 μM $MgSO_4$ + 0.01 mg/ml BSA (sigma A-8806)] (Kubota *et al.*, 2000). Thereafter, a brief electrical pulse (15 μ sec) at 2.33 kV/cm was administered using a BTX Electrocell Manipulator 2001. After the EP, the fused couplets were incubated in 5% FBS M199 medium covered with mineral oil (sigma M-8410) at 38.5℃ in 2% CO_2 till activation (Shen, 2003).

6. Activation and culture of the reconstructed embryos

After 4-5 h electrical fusion, the reconstructed embryos were activated by 5 μM calcium ionorphore (A23187) for 5 min and then immediately cultured in 2 mM 6-dimethylaminopurine (6-DMAP) for 4 h. The activated NT-embryos were then cultured in 5% FBS M199 medium at 38.5℃ in 2% CO_2 (Shen, 2003).

7. Embryo transfer

In goats, estrus synchronization of recipient doe was induced following a protocol similar to donors' doe except with 500 IU eCG (Sero-Gona®, China Chemical & Pharmacy Co., Taiwan) treatment on day 11 conjuncted with the $PGF_2\alpha$ analogue. After mid-ventral incision, the uterus was exteriorized and 1-2 cell stages embryos transferred into the oviduct ipsilateral to the ovary with more ovulation points.

In cattles, the recipient cows were derived from Holstein in natural estrus. Embryos that reached the blastocyst stage were transferred into recipient cattle approximately 7 days after synchronized estrus. One or two embryos per recipient were nonsurgically introduced into the uterine horn ipsilateral to the ovary containing a palpable corpus luteum.

Pregnancy evaluation of recipient does was performed using transrectal ultrasound at 45 days after embryo transfer, and recipient cows at 53 days (Shen, 2003).

RESULTS AND DISCUSSION

The SCNT technique in Taiwan has been successfully established since the last 3 years after the co-operation between NTU and UCONN. Results in cattle cloning showed, the enucleation rate and fusion rate of bovine NT embryos were 89% (1170/1311) and 88% (938/1064), respectively. The development rates of the NT-embryos cloned from adult ear fibroblast cells of which had been transfected with human blood clotting factor VIII (*aLA-hFVIII*) gene were 90% (670/741), 59% (384/741), and 38% (278/741) reached 2-cells, 16-cells and blastocyst stage, respectively, after cultured for 7 days *in vitro* (Table 1). One hundred and ninety-three bovine cloned transgenic embryos were transferred into 82 recipient females. Eleven of them were pregnant, with 5 giving birth to 5 cloned calves (Table 2). Unfortunately, one of the five cloned calves was dead after birth. However, none of those five survived cloned calves was evidenced harboring with the *aLA-hFVIII* transgene with southern blot analysis.

The fusion and cleavage rates of goat NT-embryos cloned from adult ear fibroblast cells with *hFVIII* gene transfected were 93% (100/107) and 23% (23/99), respectively (Table 3). One out of 11 recipients were pregnant and gave birth to 1 kid (1.1% of cloned embryos transferred) after transferring 92 NT-embryos (Table 4).

Table 1. The in vitro development of bovine NT-embryos cloned with *hFVIII* gene transfected adult ear fibroblast cells.

Enucleation Rate (enucleated/ examined)	Fusion Rate (fused/ examined)	No. of cultured NT-embryos	No.(%) of NT-embryos developed to*			
			2-cell	16-cell	CM	B
89.2 1170/1311)	87.5 (938/1064)	741	670(90.4)	384(58.5)	337(45.5)	278(37.5)

* CM: Compacted morula; B: Blastocyst.

Table 2. The results of embryo transfer with bovine NT-embryos cloned from an adult Holstein ear fibroblast cells of which transfected with *hFVIII* gene.

No of NT-embryos transferred	No. of recipients	No. (%) of recipients became pregnant	No. (%) of offspring
193	82	11 (13.4)	5 (6.14)

Table 3. The cleavage rate of goat NT-embryos cloned with *hFVIII* gene transfected adult ear fibroblast cells.

No. of NT-couplets	No. (%) of NT-couplets Fused	No.(%)of NT-embryos cultured	No. (%) of NT-embryos developed to 2-cell after culture
107	100(93)	99	23(23)

Table 4. The results of embryo transfer with goat NT-embryos cloned from an adult Alpine ear fibroblast cells of which transfected with *hFVIII* gene.

No of NT-embryos transferred	No. of recipients	No. (%) of Recipients Became pregnant	No. (%) of offspring
92	11	1 (9.1)	1 (9.1)

Moreover, the kid was evidenced harboring with the *aLA-hFVIII* transgene when their genomic DNA had been subjected to the southern blot analysis.

In this study, the cloning efficiency was evaluated based on the number of cloned offsprings produced over the number of embryos transferred. The overall cloning efficiency in bovine and goat were 2.6% (5/193) and 1.1% (1/92), respectively. It suggested that the cloning efficiency of bovine and goat were still low in Taiwan. It is known that the cloning efficiency is closely associated with epigenetic reprogramming (Kang *et al.*, 2001; Xue *et al.*, 2002). Despite the recent successes in cloning various animal species, using somatic cells as the source of donor nuclei has raised many practically relevant problems such as increasing abortion rates, high birth weight

and perinatal death (Campbell *et al.*, 1996; Wilmut *et al.*, 1997; Schnieke *et al.*, 1997). The production of viable offspring derived from NT depends on the successful combination of numerous factors including the quality and age of recipient oocytes, the procedures for reconstruction of the NT embryos, the culture conditions, as well as the cell cycle stages of both the donor and recipient cells (Campbell, 1999).

Calves and lambs resulting from embryos produced in vitro may be heavier at birth weight than that produced from *in vivo* (Willadsen *et al.*, 1991; Keefer *et al.*, 1994; Behboodi *et al.*, 1995; Sinclair *et al.*, 1995; Walker *et al.*, 1996) and increased incidences of neonatal mortality (Schmidt *et al.*, 1996; Kruip and den Daas, 1997). These phenomena might be attributable to the suboptimal culture conditions such as serum in the medium or the manipulation procedure itself (Garry *et al.*, 1996; Kruip and den Daas, 1997). In this study, reconstructed goat embryos transferred into the oviducts of the recipients during 1- to 2-celled stages, but the bovine NT embryos were transferred during blastocyst stage after *in vitro* culture for 7 days. Therefore, the goat NT embryos only exposed to a relatively short period of *in vitro* culture compared to bovine NT embryos. This may have explained why the cloned kid healthy grew, but one of five cloned calves was died after birth. On the other hand, the post-neonatal death occurred in our cloned calf might due to those processes for adopting to the nuclear transfer adopted. The major problem might be resulted from the quality of those recipient oocytes employed. The fact of using poor quality of oocytes as the recipient cytoplasts might have impairments of both developmental capacity and gene expression in those embryos reconstituted after nuclear transfer. Also, some inappropriate epigenetic-reprogramming events might have therefore been resulted (Kang *et al.*, 2001). Unfortunately, the ovaries obtained from abattoirs in Taiwan were usually provided from the cows with infertility syndrome which might have consequently resulted in poor quality of oocytes essentially used in our previous studies.

Whether the pregnancy rate after the embryo transfer is consistent with the production rate of cloned calf remains to be verified. After all, the number of genes to be turned on during the single cell's blastocyst stage is much less than the number of cells needed for a grown-up. In the development process of cloned embryo, the bottleneck facts on both the high fetus loss after pregnancy and the low postnatal survivability after birth are much tougher than the development ability before the preimplantation of embryos (Heyman *et al.*, 2002). Therefore, the cow embryos cloned in Taiwan with a satisfactory development to blastocyst stage in vitro may not necessarily mean that they possess the same developmental potentiality to culminate normal offspring born after re-implantation to the recipients. Using the *ovum pick up technique* to collect the *in vivo* maturation of bovine oocytes as recipient cell might be able to solve the problem partially. However, there is no explanation for the relationships between the cloned died calf and the inferior quality of recipient oocyte.

A number of related problems such as the early losses of somatic cell cloned embryos, fetus losses, larger offspring syndrome, inferior adaptation, and postnatal mortality has been studied (Campbell *et al.*, 1996; Wilmut *et al.*, 1997; Schnieke *et al.*, 1997; Wells *et al.*, 1999; Cibelli *et al.*, 1998). Some researchers believe that these issues were resulted from nuclear transfer process, reprogramming of donor nuclei, varied donor cells and *in vitro* culture system as Wells *et al.* (1998) mentioned in their report. As far as the nuclear transfer animals concerned, the abnormal situations developed by the maternal placental are often the main cause for the embryos early losses (Stice *et al.*, 1996); and the results in hydrallantois, enlarge placenta and umbilicus, and placental abnormality in the later gestation (Kruip and den Daas, 1997). As a result, the probability of neonatal is approximately one fourth of the *in vitro* produced embryos of *in vitro* maturation and *in vitro* fertilization (Wells *et al.*, 1998). Heyman *et al.* (2002) found that the abortion and the prthologic placentation of cloned calves were probably highly related to the pregnancy serum protein 60 (PSP60) secreted by the recipient. They indicated that the excessive PSP60 found in the blood of recipient female of the cloned bovine in the early gestation contributed to the fetus losses at early or later gestation of the cloned bovine. They also indicated that hydrallantois and enlarge placenta were found in the planceta of cloned bovine. Recently, the research on the embryonic gene expression of nuclear transfer indicated that the expression profiles of 3 of 6 genes in the early embryonic development stage were significantly different from the embryo produced through *in vitro* fertilization (Daniels *et al.*, 2000). As shown in the results of methylation level of specific DNA fragment, the specific DNA fragment was highly methylated in the somatic cell before the nuclear transfer process. The nuclear transfer embryo produced after nuclear transfer was highly methylated. On the other hand, the embryo produced by the specific DNA fragment through *in vitro* fertilization was slightly methylated. The results suggested that the recipient cell was unable to reprogram the genome and, consequently, the nuclear transfer embryo produced by the SCNT showed an inferior development ability. To the worst extent, the cloned calves became abnormal and the postnatal survivability dropped to a low level (Humpherys *et al.*, 2001; Kang *et al.*, 2001).

Although, we have generated one cloned transgenic goat, but all of five cloned calves were not harboring with the *aLA-hFVIII* transgene in this study. This was the same as results from other laboratory, which showed some animals generated by nuclear transfer from transfected cells failed to be transgenic. It is mostly due to the bystander effect of the transfected cells occurring in the selection process after foreign gene transfection, where transgenic cells, which express the antibiotic-resistance gene, provide protection to nearby nontransgenic cells either by secretion of the gene product into the medium or by direct cell-to-cell contact (Betthauser *et al.*, 2000; Bondioli *et al.*, 2001; Denning *et al.*, 2001; Echelard *et al.*, 2002; Lai *et al.*, 2002). Owing to the possible bystander effect in this study, the transfected donor cells might mixed and contain both transgenic and nontransgenic cells.

In conclusion, this study has confirmed that SCNT is a viable technique for bovine (Fig. 1) and goat (Fig. 1) cloning and produced one cloned-transgenic goat successfully. However, it is failed for obtaining a complete sequence of *hF VIII* transgene in the genome of the transgenic cloned calves. Further studies will be

required to gain those particular clones of donor cells harboring the intact transgene before being subjected to the nuclear transfer to ensure the desired transgenic offspring successfully cloned, and to evaluation the low cloning efficiency whether due to caused by incomplete reprogramming of the foreign nuclear materials or other factors.

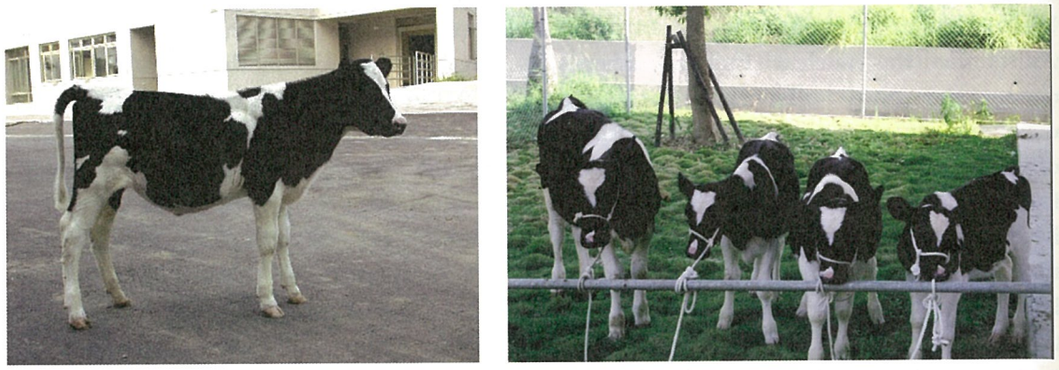

Fig. 1. Five cloned calves produced from an adult Holstein ear fibroblast cell of which transfected with *hFVIII* gene.

Fig. 2. A cloned-transgenic goat produced from adult Alpine ear fibroblast cell of which transfected with *hFVIII* gene.

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The Immune Response of Taiwan Abalone *Haliotis diversicolor supertexta* and Its susceptibility to *Vibrio parahaemolyticus* under Environmental Stress

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ABSTRACT

Taiwan abalone held in 30 ‰ seawater and then transferred to 20, 25, 30 and 35 ‰ were examined for immune parameters, and phagocytic activity and clearance efficiency to *Vibrio parahemolyticus* after 24 and 72 h. The THC (total haemocyte count) increased directly with salinity levels. Phenoloxidase activity, respiratory burst, phagocytic activity and clearance efficiency to *V. parahaemolyticus* decreased significantly for the abalone transferred to 20, 25 and 35 ‰. Abalone held in 30 ‰ seawater and 28℃ and then transferred to 20, 24, 28 and 32℃ were examined for immune parameters after 24 and 72 h. The phenoloxidase activity and phagocytic activity decreased significantly for the abalone transferred to 32℃, whereas the THC and respiratory burst increased significantly for the abalone transferred to 32 ℃. The abalone showed decreased immunity and decreased resistance against *V. parahaemolyticus* infection when the animals were subjected to stress both in salinity and temperature.

Key words: Immune response, *Haliotis diversicolor supertexta*, Susceptibility, *Vibrio parahaemolyticus*, Environmental stress.

INTRODCTION

Temperature and salinity are the primary physical factors affecting the life of molluscs. Salinity in the range of 30-35 ‰ and temperature in the range of 24-30℃ are the optimal levels for the growth of Taiwan abalone (Chen, 1984). It is known that Taiwan abalone reared at 30 ‰ and at 20, 25 and 30℃ survive temperature in the range of 4.5-33.1, 7.7-33.7 and 13.5-36.5℃, respectively when water temperature was decreased or increased gradually (Chen and Chen, 1999). Twiwan abalone reared at 30 ‰ and at 20, 25 and 30℃ survive salinity in the range of 18-39, 16-37 and 15-36 ‰, respectively when salinity was decreased or increased gradually (Chen and Chen, 2000).

In molluscs, agranular haemocytes (hyalinocytes) and granular haemocytes (granulocytes) are involved in phagocytosis (Bayne, 1990). Haemocytes are also involved in coagulation and the production of melanin via the prophenoloxidase system (Söderhäll *et al.*, 1996). During phagocytosis, the release of superoxide anion and other ROIs (reactive oxygen intermediates) is known as respiratory burst, and it plays an important role in microbicidal activity.

Taiwan abalone has been mostly cultured in land-based concrete farms adjacent to the sea coast. The salinity in abalone farms may decrease suddenly to as low as 20 ‰ during the rainy season and when typhoons strike. Seasonal range of water temperature in fish farms may vary from 15 to 32℃. Culture of abalone has suffered mass mortality due to vibriosis since 1998 in Taiwan. The bacteria, *Vibrio parahaemolyticus* and *V. alginolytius* isolated from the haemolymph of moribund abalone, have been demonstrated to cause outbreaks of vibriosis associated with warm temperature (Lee *et al.*, 2001).

This study was aimed at determining the immune response of Taiwan abalone under salinity change and temperature change. Total haemocyte count (THC), phenoloxidase activity, respiratory burst (release of superoxide anion), and phagocytic activity of abalone to *V. parahaemolyticus* were examined. Challenge tests were also conducted.

MATERIALS AND METHODS

Taiwan abalone were obtained from a commercial farm were acclimated at 30 ‰ for two weeks at room temperature (26-28 ℃). During the acclimation period, animals were fed red seaweed *Gracilaria tenuistipitata* daily.

V. parahaemolyticus was isolated from diseased abalone. It was cultured on 50 ml tryptic soy broth supplemented with 3 % NaCl (TSB, Difco) for 24 h at 30℃. The stock cultures were centrifuged at 7155 x g for 15 min at 4℃. The supernatant fluid was removed and the bacterial pellet was re-suspended in saline solution at 8 x 10^6 and 8 x 10^8 cfu ml^{-1} as stock bacterial suspension for injection challenge, and the tests of phagocytic activity and clearance efficiency.

Each abalone was injected into the pallial sinus with 20 µl of bacterial suspension (8 x 10^6 cfu ml^{-1}). Challenge tests at a dose of 1.6 x 10^5 cfu $abalone^{-1}$ were conducted in triplicates. The test and control groups comprised 10 abalones each. After injection, abalones were kept in 15 l tanks (10 abalones in each) containing 10 l of aerated water at 20, 25, 30 (control) and 35 ‰. Abalones injected with an equal volume of sterile saline solution and kept in 30 ‰ seawater served as unchallenged controls (Table 1).

For the challenge test on temperature stress, abalones were kept in 15 l tanks (10 abalones in each) containing 10 l of aerated seawater at 20, 24, 28 and 32℃ after injection. Abalones injected with an equal volume of sterile saline solution and kept in 28℃ served as the unchallenged controls (Table 2).

For haemocyte counts and enzyme activity assays, abalones acclimated to 30 ‰ seawater were placed in eight replicates in aquaria with 20, 25, 30 and 35 ‰ seawater that was renewed daily for 5 days. Water temperature was maintained at 26 ± 1℃ (Cheng *et al.*, 2004a). For the temperature stress, abalone acclimated to 30 ‰ seawater at 28℃ were transferred to 20, 24, 28 and 32 ℃. Abalones were sampled individually at the beginning of the test and at 24, 72 and 120 h (Cheng *et al.*, 2004b).

Haemolymph was withdrawn. A drop of diluted haemolymph (50 µl) was placed on a haemocytometer, and THC was measured using an inverted phase contrast microscope. Phenoloxidase activity and respiratory burst were measured.

For phagocytic activity and bacterial clearance tests, abalones after 0, 24, 72 and 120 h exposure at 20, 25, 30 and 35 % and after exposure to 20, 24, 28 and 32 C were injected with 20 µl bacterial suspension (8 x 10^8 cfu ml^{-1}

and 5 x 10^8 cfu ml^{-1}), resulting in 1.6 x 10^7 and 1 x 10^7 cfu $abalone^{-1}$ for salinity stress test and temperature stress test, respectively. After injection, the abalones were held in their respective test solutions for 3 h. Two hundred microliter of haemolymph were collected from the pallial sinus.

Phagocytic activity was measured. Two hundred haemocytes were counted and the phagocytic rate (PR) was expressed as follow:

PR={(Phagocytic haemocytes)/(Total haemocytes)} x 100

Clearance efficiency was measured. Clearance efficiency to *V. parahaemolyticus* was calculated and expressed as percentage inhibition (PI) as follows:

PI = 100- {(cfu in test group)/(cfu in control group)} x 100

A multiple comparison (Tukey) test was conducted to compare the significance of differences among treatments using the SAS computer software (SAS Institute Inc., Cary, NC, USA).

RESULTS

All the unchallenged control abalones survived. By contrast, the onset of mortality occurred at 6 h in the challenged abalone held at a salinity of 20, 25 and 35 ‰. Over 24-120 h, the cumulative mortality for the abalone transferred to 25 ‰ and 35 ‰ was significantly higher than for the abalone held at 30 ‰ seawater (Table 1).

All the abalones transferred to temperature of 32℃ died within 72 h. Over 24-120 h, the cumulative mortality for the abalone transferred to 32℃ was significantly higher than for the abalone held at 28℃ (Table 2).

Table 1. Susceptibility of Taiwan abalone to *Vibrio parahaemolyticus* at different salinity levels at 26±1℃. Data in the same column with different letters are significantly different ($P<0.05$) among treatments. Values are mean±SE (n=30 abalones in each case).

Bacterial dose	Salinity	Cumulative mortality (%)						
(cfu $abalone^{-1}$)	(‰)	6	12	24	48	72	96	120
Control	25	0	0	0	0	0	0	0
1.6×10^5	20	26.7 ± 3.3^a	40.0 ± 5.8^a	70.0 ± 5.8^a	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a
1.6×10^5	25	10.0 ± 5.8^b	33.3 ± 3.3^{ab}	46.7 ± 3.3^b	56.7 ± 3.3^b	63.3 ± 3.3^b	63.3 ± 3.3^b	63.3 ± 3.3^b
1.6×10^5	30	0.0 ± 0.0^b	3.3 ± 3.3^b	20.0 ± 5.8^c	23.3 ± 3.3^c	36.7 ± 3.3^d	36.7 ± 3.3^d	36.7 ± 3.3^d
1.6×10^5	35	3.3 ± 3.3^b	20.0 ± 5.8^b	40.0 ± 5.8^b	50.0 ± 0.0^b	53.3 ± 3.3^c	53.3 ± 3.3^c	53.3 ± 3.3^c

Table 2. Susceptibility of Taiwan abalone to *Vibroio parahaemolyticus* at different temperatures at 30 ‰. Data in the same column with different letters are significantly different ($P< 0.05$) among treatments. Values are mean ±S.E (n=30 abalones in each case).

Bacterial dose	Temperature	Cumulative mortality (%) time after challenge (h)					
(cfu $abalone^{-1}$)	(˚C)	6	12	24	48	72	96
Control	28	0	0	0	0	0	0
1.6×10^5	20	0.0 ± 0.0^b	10.0 ± 5.8^c	23.3 ± 3.3^c	33.3 ± 3.3^c	36.7 ± 6.7^c	36.7 ± 6.7^c
1.6×10^5	24	0.0 ± 0.0^b	16.7 ± 6.7^b	23.3 ± 3.3^c	30.0 ± 5.8^c	33.3 ± 6.7^c	33.3 ± 6.7^c
1.6×10^5	28	3.3 ± 3.3^{ab}	26.7 ± 6.7^b	33.3 ± 6.7^b	53.3 ± 3.3^b	60.0 ± 5.8^b	60.0 ± 5.8^b
1.6×10^5	32	10.0 ± 5.8^a	33.3 ± 3.3^a	56.7 ± 3.3^a	76.7 ± 3.3^a	100.0 ± 0.0^a	100.0 ± 0.0^a

After 24 h, the THC decreased significantly by 49 % and 31 % for the abalone transferred to 20 and 25 ‰ salinity, respectively. After 24 h, phenoloxidase activity decreased significantly for the abalone transferred to 20, 25 and 35 ‰. The respiratory burst decreased significantly for the abalone transferred to 20, 25 and 35 ‰. After 24 h, phagocytic activity decreased significantly to 9 %, 11 % and 13 %, and clearance efficiency decreased by 66 %, 59 % and 72 % for the abalone transferred to 20, 25 and 35 ‰, respectively, compared to the abalones held in 30 ‰.

After 24 h, the THC increased significantly by 22 % and 29 % for the abalone transferred to 20 and 32 ℃, respectively. Phenoloxidase activity decreased significantly for the abalone transferred to 24 and 32 ℃, whereas phenoloxidase activity increased for the abalone transferred to 20℃ after 24, 72 and 120 h. The respiratory burst decreased significantly for the abalone transferred to 20 ℃ after 24 h, whereas the respiratory burst increased significantly for the abalone transferred to 32℃ after 72 and 120 h. After 24 h, phagocytic activity decreased significantly to 10 %, 13 % and 9 % for the abalones transferred to 20, 24 and 32 ℃, respectively, as compared to the activity of abalones at 28℃.

DISCUSSION

Taiwan abalone was more susceptible to *V. parahaemolyticus* when the animals were transferred to 20 and 25 ‰ from 30 ‰, and when the animals were transferred to 32℃ from 28℃. We may conclude that change in temperature and salinity can trigger disease outbreak by affecting the defense mechanism of the host: the susceptibility of abalone to *V. parahaemolyticus* is enhanced by high temperature (32℃) and low salinity (20 and 25 ‰). All the abalones transferred to 20 ‰ were killed in 48 h. Taiwan abalone maintained in 30 ‰ and at 20, 25 and 30℃ exhibited 96-h LS50 (median lethal salinity) of 22.1, 20.8 and 19.9 ‰ when the abalone were suddenly transferred to a series of low salinity levels (Chen and Chen, 2000). Taiwan abalone which had been maintained in 30 ‰ and at 30℃, and transferred to a series of higher temperature, exhibited 24-h LT50 of 32.2℃ which was higher than the LT50 of present study (31.1℃) (Chen and Chen, 1999)

The total number of circulating haemocytes can be affected by environmental change. Sâo Paulo shrimp *Farfantepenaeus paulensis* reared in 34 ‰ had a significantly higher THC (20 % more) as compared to the shrimp reared at 22 and 13 ‰ (Le Moullac and Haffner, 2000). *M. rosenbergii* reared in 15 ‰ had a significantly higher THC (15 % more) when compared to prawns reared in freshwater (Cheng and Chen, 2000). Similar phenomenon was observed in the present study: the THC of Taiwan abalone increased by 44 % and 56 % for the abalone reared in 30 and 35 ‰, respectively, as compared to the abalone reared in 25 ‰. *M. rosenbergii* reared in 27 and 30℃ had a significantly higher THC as compared to prawns reared in 20 and 33℃. An increase of circulating haemocyte count occurred in the Pacific oyster *Crassostrea gigas* following 15 min mechanic disturbance (Lacoste *et al.*, 2002). The fact that the THC increased for the abalone transferred to 20℃ is considered a consequence of stress response.

In *M. rosenbergii*, phenoloxidase activity, phagocytic activity and clearance efficiency

to *L. garvieae* were significantly higher for animals reared at 5 and 10 ‰ than those reared in freshwater and 15 ‰ (Cheng and Chen, 2000; Cheng et al. 2003). In the present study, the increases of both phenoloxidase activity and respiratory burst are well correlated with the number of THC for abalone in the range of 20-30 ‰. In *M. rosenbergii*, phenoloxidase activity, phagocytic activity and clearance efficiency to *L. garvieae* were significantly higher for animals reared at 27 and 30℃ than those reared in 20 and 33℃. In the present study, phenoloxidase activity increased together with an increase in THC for the abalones transferred to 20℃.

Phagocytosis is affected by environmental parameters in invertebrates (Bayne, 1990). For example, an elevated temperature has been reported to increase phagocytosis (Feng and Feng, 1974) and haemocyte activity (the ability of haemocyte to adhere the fluorescent beads) in the American oyster *C. virginica*. Decrease in salinity has been reported to increase haemocyte activity in *C. vurginica* and in European flat oyster *Ostrea edulis* (Fisher *et al.*, 1987). A reduction in phagocytosis was observed for *Haliotis rufescens* in 35 ‰ and 45 ‰ (Martello *et al.*, 2000). In the present study, phagocytic activity and clearance correlated with increased susceptibility of abalone to *V. parahaemolyticus* when the abalone were transferred to 20, 25 and 35 ‰ from 30 ‰ in 72 h. The phagocytic activity of *V. parahaemolyticus* decreased for the abalone transferred to 20 and 32 ℃ in 24 h.

In conclusion, the susceptibility of abalone to *V. parahaemolyticus* correlated with reductions in immune parameters including phenoloxidase activity, respiratory burst, phagocytic activity and clearance efficiency when the abalone were transferred to 20, 25 and 35 ‰ from 30 ‰ in 72 h. Taiwan abalone transferred from 28 ℃ to 32 ℃ showed higher susceptibility to *V. parahaemolyticus*, and lower phenoloxidase activity and phagocytic activity indicated a reduction in immune ability.

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Control of Avian Reticuloendotheliosis

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ABSTRACT

Reticuloendotheliosis (REV) infection is common, but not ubiqutous. In order to understand the REV infection status, antibody determination and virus isolation were performed for chickens in Taiwan. The results showed that REV infection is quite common in chickens. Two REV viruses were isolated from infected chickens and one from a fowl poxvirus vaccine. The phylogenetic analysis of those REV isolates will be presented.

Key words: gp85 protein, Monoclonal antibody, Subgroup J avian leukosis virus, Surface plasmon resonance.

INTRODUCTION

Reticuloendotheliosis viruses (REVs) belong to the Gammaretrovirus genus and have a genomic structure consisting of the group-specific antigen (gag), protease (pro), polymerase (pol), and envelope (env) regions flanked by long terminal repeats (LTRs). Birds infected with REV after hatching, a transient viremia followed by antibody formation. Infection of older birds rarely results in clinical diseases (Witter, 1985).The prevalence of seropositive flocks and the proportion of seropositive birds in a flocks increase with birds' ages. In certain instances, some infected chickens show chronic lymphomas and an immunosuppressive runting disease. Two types of tumors are found in infected chickens, bursal and non-bursal. The bursal type tumor originates in the cloacal bursa, and later spreads to liver and other organs in 17-43 weeks after inoculation with nondefective chick syncytial or T strains of REV (Witter, 1981). A second type of chronic tumor, nonbursal lymphoma, in chickens occurs by 6 weeks after inoculation with spleen necrosis or chick syncytial strains of nondefective REV (Witter, 1986). Some birds show an immunosuppressive runting disease, this developed by 3-5 weeks after infection (Mussman, 1971).

Although REV infection is common, natural outbreaks of this disease in commercial poultry are rare. One possibility is from contaminated vaccines, especial fowl poxvirus or Marek's disease virus vaccines (Fadly, 1996; Koyama, 1976). Those vaccines have been used extensive in chickens. The impact of vaccination and the relationship between infection and vaccination are not clear for the present. This paper describes the REV infection status in chickens and compares the REVs from infected chickens and vaccines.

MATERIALS AND METHODS

Case history

Case 3122/03 was from a breeder farm in Yilan with two kinds of breed, breed A and breed B, 3 flocks each. The ages and the breed of those chickens in this farm were 10, 38, 66 weeks old of breed A and 13, 39, 63 weeks old of breed B in December 2003. Besides ordinary vaccination for commercial broiler breeder chickens, those chickens were vaccinated with live herpes virus of turkey and live SB1 strain of Marek's disease virus vaccines at one day of age and vaccinated two times with live fowl poxvirus vaccines at the age of 1 and 13 weeks of age, respectively. Those chickens were considered to be normal because their weekly mortality ranged from 0 to 0.15%. Blood samples were taken from those chickens for REV antibody determination and virus isolation.

Case 3295/04 was from a color breeder farm with 15,000 chickens. The weekly mortality of this farm was less than 0.2%, considered to be normal. The blood samples were taken for REV antibody determination and virus isolation.

REV antibody determination

Plasma samples from different flocks were tested for the presence of anti-REV antibody by using a commercial ELISA kit (Idexx, Westbrook, ME) according to the manufacturer's instructions.

REV virus isolation from chickens

Blood samples were taken from Whole blood was collected with heparin (Becton Dickinson, Franklin Lakes, NJ) and put on ice immediately. Buffy coat from whole blood was co-cultured with DF1 cells (ATCC CRL-12203, American Type Culture Collection, Manassas, VA) and incubated at 39 C for 7 days each passage. The presence of virus was examined by RT-PCR in supernatant with LTR primers for long terminal repeat (Aly, 1993). Virus isolation was attempted three passages in cell culture before considering negative.

REV gene detection in vaccines by PCR

Eighteen commercial fowl poxvirus vaccines and 18 Marek's disease vaccines sent for inspection at Animal Health Research Institute, Taiwan were collected for REV gene detection in 2003 and 2004, respectively. The REV LTR gene in fowl poxvirus vaccines was with primer, TR-1/TR-2 (Singh, 2003) and that in Marek's disease vaccines was detected by polymerase chain reaction by using LTR primer (Aly, 1993).

REV virus isolation from vaccines

All the collected vaccines were re-suspended with PBS and co-cultrued with DF1 cells. After changing medium at the second day, those cells were culture for 1 week in one passage. The presence of virus was examined by RT-PCR in supernatant with LTR primers for long terminal repeat (Aly, 1993). Virus isolation was attempted three passages in cell culture before considering negative.

REV env gene sequencing

The env genes of those REV isolates were amplified by RT-PCR with env5/env6 primer (new designed), amplifying the whole env gene and cloned into yT&A vector (Yeastern Biotech, Taipei, Taiwan) and sequenced.

RESULTS

The seropositive prevalence and virus isolation in the broiler breeder farm

The seropositive prevalence and the ages of case 3122/03 farm in breed A were 0% (0/16) at the age of 10 weeks, 24% (4/17) at the age of 38 weeks, and 93% (15/16) at the age of 66 weeks and those in breed B were 0% (0/13) at the age of 13 weeks, 92% (12/13) at the age of 39 and 100% at the age of 63 weeks. The seropositive prevalence increased with ages but not immediately after Marek's disease or fowl pox vaccinations. A REV, 3122/03, was isolated from a 31-week-old female broiler breeder chicken of breed A from that farm. The total virus isolation rate from those chickens was 1% (1/88).

The seropositive prevalence and virus isolation in the color breeder farm

The seropositive prevalence and the ages of case 3259/04 farm were 0% (0/15) at the age of 12 weeks, 93% (13/14) at the age of 25 weeks, 67% (10/15) at the age of 26 weeks, 86% (12/14) at the age of 28 and 100% at the age of 50 weeks. One REV virus, 3295/04, was isolated from a 12-week-old chicken.

Detection and virus isolation of REV in vaccines

Seven of the tested 18 fowl poxvirus vaccines had approximate 290 bp PCR product. However, only one REV was isolated from those vaccines. The vaccine with positive virus isolation had not this PCR band. No PCR product was found in Marek's disease vaccines with the primer used. After two passages of virus isolation from fowl poxvirus vaccines in DF1 cells, a faint band was found. After 13 times of passage, the PCR became more clear and finally a REV, 3134/03 was isolated from that vaccine.

REV env gene analysis

The identity of the env genes from the two chicken REV isolates, 3122/03 and 3259/04, was 99.7%. The REV isolate, 3134/03, from fowl poxvirus vaccine showed 461 n.t. deletion in its env gene when compared with the chicken isolates. Thus, the identities of this REV and those two chicken isolates were 67% and 67.1% respectively. Another clones from the same virus will be performed for this deletion. The identities of a reference strain, FPV-REV (AF246698) and these chicken isolates were 99.7% and 99.8%, respectively. The identity of strain A REV with the present two chicken isolates (X01455) was 95.5%.

DISCUSSION

Although REV infection is common but not ubiquitous (Witter and Fadly, 2003). Data presented in this paper document the presence of REV in the field is quite common. Almost hundred percent of chickens get infection at old ages. However, those chickens showed neither clinical signs nor REV-induced tumors. Like avian leucosis virus infection, chicken showed resistant to REV infection after 1 week of age (Witter and Fadly, 2003). From the late seroconversion of those flocks (after 16 weeks old), those chickens were infected at the later ages. This means those chickens established their resistance but not immune tolerance getting infection before hatching.

Infection of those chickens with REV was not caused by contaminated vaccines since the santibody didn't appear immediately after Marek's disease and fowl pox vaccinations. In addition, the low prevalence at the beginning of sero-conversion (at the age 20 weeks, 7 weeks after the second fowl pox vaccination) revealed some chickens got infection at the beginning because vaccination likely caused almost the whole seroconversion but not only some.

More than 1/3 of fowl poxvirus vaccines contained small part of REV LTR gene and failed to give virus in culture. This means a small LTR fragment remains in the vaccine genes but fail to produce intact virion. REV LTR incorporated in the genomes of fowl poxvirus vaccines without indication of the presence of infectious REV (Moore, 2000). Large part of the REV provirus may be lost from host DNA over time, whereas LTRs remaining (Robinson, 1986).

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Production of Rapid Growth and Specific Pathogen Resistant (SPR) Broodstock of *Litopenaeus vannamei* and Application of β-Glucan on Shrimp Aquaculture

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ABSTRACT

The present study attempted to establish a system for the production of rapid growth and specific pathogen resistant (SPR) broodstocks of Pacific white shrimp (*Litopenaeus vannamei*). The studies were focalizing on the production of white spot disease virus (WSSD) resistant shrimps. The results obtained from the present study showed that shrimps derived from WSSD resistant broodstocks revealed a significantly higher survival rates than those of the control shrimps. It was also showed that shrimp obtained from rapid growth strain of *L vannamei* showed a better growth rates than those of the regular shrimp. The study on the investigation of efficacy of β-Glucan produced from various fungi including *Schizophyllum commune* and *Ganoderma lucidum* and *Agaricus blazei* showed a significant contribution on the survival of cultured *L. vannamei*.

Key words: Repid growth, β-Glucan, Survival, Growth, *Litopenaeus vannamei*.

INTRODUCTION

Following culture techniques of Pacific white shrimp (*Litopenaeus vannamei*) has been successfully established in Taiwan in the year of 1998, this species has become one of the most important cultured shrimps in Asia. In the year of 2001, the production for this species upgraded to 100 thousand and 10 thousand metric tonnes in China and Taiwan respectively. It is anticipated that the production for *Litopenaeus vannamei* will significantly increase up to 2,000-3,000 thousand and 200 thousand metric tons in these two areas in the year of 2005. For sustainability of Pacific white shrimp culture, developing techniques for a stable production and preventing the occurrence of diseases for this species are considered to be the most important. Amongst various factors that affect the stable production of Pacific white shrimp, elimination of viral infectious diseases and uses of fast growth seed were recommended as key factor to affect the success of *L. vannamei* culture. There is an urgent need in developing systems for culture of specific pathogen resistant (SPR) and fast growth strain of *L. vannamei*.

The present study attempts to develop a system for growing or selecting of rapid growth and specific pathogen resistant (SPR) Pacific white shrimp (*Litopenaeus vannamei*).

It is hoped that through the accomplishment of the study, the technologies for SPR shrimp may effectively developed in Taiwan so that sustainability of the shrimp aquaculture will become possible. The present study also aims to investigate the potentiality for the SPR and fast growth shrimps.

Growing shrimps are usually under the stresses from environment, food, the excessive culture density of animal itself and the challenge of the pathogens. Diseases of cultured shrimps may cause mass economic losses. To obtain a better survival and harvesting for shrimp, uses of drugs and vaccines is the only strategy in the past. However, in recent several years, drug or vaccines become less effective and over uses of synthetic chemicals has already threaten to the human health. In a meeting entitled "Monitoring anti-microbial usage in food animals for the protection of human health" held in 2001 in Oslo, Norway by World Health Organization (WHO) strongly suggested that uses of drug for food animals should be stickly regulated. Due to public health problem arisen from contaminated toxic chemicals in food animals, many countries have used drug residue existing in food animals as a trade barrier. For this reason, shrimp culture industry is considered to be a very high risk investment. It is therefore urgently needed to look for a non-toxic and total safe strategies for the industry.

Following long term investigation, it is concluded that uses of natural immunostimulant is one of the best strategies to ensure a sufficient survival rate as well as a better quality of product. Amongst a very few immunostimulant used it is demonstrated that β -Glucan especially β 1,3, β 1,6-D-Glucan may effectively enhance the natural defenses of domestic and aquatic animals against all kind of negative challenges. The present study therefore attempts to investigate the efficacy of β -Glucan on immune-enhancing activities through evaluation of hemocyte variation in hemolymph and variation of superoxide disunities (SOD) in hepatopancreas was also evaluated. The survival of culture shrimps (*Litopenaeus vannamei*) following β -Glucan ingestion was also evaluated.

MATERIALS AND METHODS

The original seeds of experimental shrimps (*Litopenaeus vannamei*) used in the present were obtained from Hawaii USA. For the evaluation of specific pathogen resistace (SPR) and rapid growth, 2nd generation obtained from the pond infected with WSSD were selected. After mass motality due to WSSD, the existing shrimps were continuously cultured for 6 months then the groups of biggest shrimps were collected for the hatchery and experimental uses. All the larvae obtained from the above described strains were cultured in a 30-liter aquaria than the growth rates and the challenge infection by feeding of white spot diseased shrimp was performed. The growth rate was calculated by weighting the body weight at the 4, 8, 12, 13, 16 and 20 weeks after cultivation. The capability of white spot disease resistant of *L. vannamei* was performed by calculating survival rates at three week after feeding of white spot disease shrimp muscles. The shrimps obtained from regular hatchery using the broostocks obtained from local cultured ponds were used as control experiment. A recepis of artificial shrimp feed as desoribed in Table 2 was designed and tested for the efficacy on the enhance of maturation of shrimp.

To evaluate the efficacy of β -Glucan variation of hemocyte and superoxide dismutase (SOD) activity following ingestion of β -Glucan were performed. β -Glucan derived from mushrooms including *Schizophyllum commune*, *Ganoderme lucidum* and *Agarics blaze* at a concentration level of 70% was supply by Perfect Health Biotechnology Co. Ltd, in Taiwan. For comparison, sodium alginate and laminarin (extracted from *Laminaria digitata*) obtained from Sigma, U.S.A were used.

The experiments were divided into 21 groups each group contains 40 shrimps. Each group feeded with feed containing 0, 0.1% or 0.4% of β -Glucan, sodium aginate and laminarin respectively. 0, 1, 2, 4, 8, 16, 30 days following ingestion of β -Glucan, sodium aginate and laminarin, 5 shrimps were sacrificed for each group at each time interval. The samples of hemolymph was collected and hemocyte in hemolymph was also counted. For SOD examination, hempatopancrease from sacrificed shrimps were also collected.

The collected hepatopacrease was processed for superoxide dismatase, SOD assay using RANSOD Kit (Randox).

To evaluat the efficacy of β-Glucan in the field, β -Glucan (70% concentration) supplied by Perfect Health Biotechnology, Co. Ltd in Taiwan was used as feed additive at concentration level of 0.5%. Three ponds containing approximately 3-5$\times 10^4$ shrimps were feeded with the obove experimental feed. No β-Glucan feed was used as control experiments. Following 5 month cultured periods, survival and growth rates are calculated for both experimental and control ponds.

RESULTS

Results presented in Table 1 and Fig.1 showed that the larval shrimps of *L. vannamei* obtained from SPR broodstocks showed a significantly higher survival rates than those of the control shrimps. It is suggested the broodstock collected from SPR selected broodstock may show a very significant resistance against white spot disease virus. It is also showed that the larvae derived from broodstock of bigger size may reveal a rapid growth rates than those of derived from the broodstock in regular size.

It may suggest that the selection of size and SPR broodstocks is impartant for *L. vannamei* aquaculture.

To achieve a complete aquaculture systems for Pacific white shrimp (*Litopenaeus vannamei*), artificial broodstock feed as deseribe in Table 2 was used for the availability of mature broodstock of *L. vannamei*. The results showed that using of the artificial broodstock feed containing the described ingredients, an enhancement of maturation for *Litopenaeus vannamei* can be achieved.

Table 1. Survival of white spot disease resistant shrimps and control after challenge of pathogen.

	Experiment No.	Survival No.	Survival Rates
Control Group A	1000	352	35%
Control Group B	1000	283	28%
SPR A	1000	724	72%
SPR B	1000	651	65%

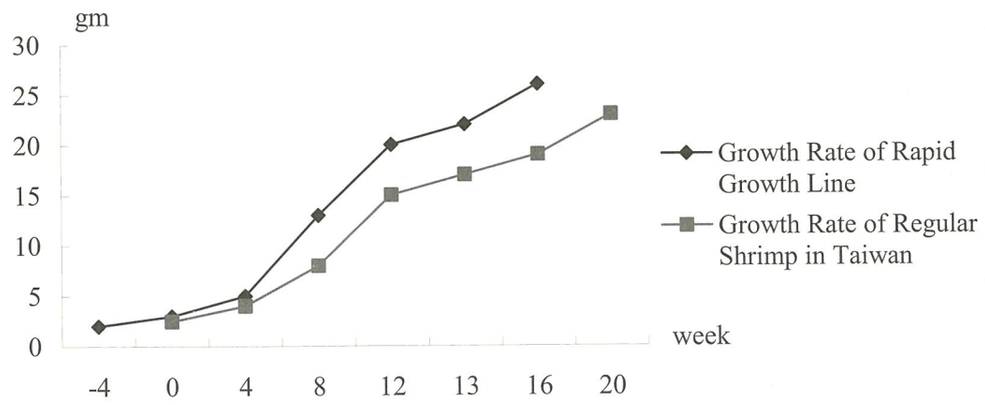

Fig.1. Growth Rates of Selected Rapid growth shrimps and the Control shrimps.

Table 2. Main Ingredients for Artificial Broodstock Feed of *Litopenaeus vannamei.*

Ingrechinet	%	Ingrechinet	%
shrimp meal	10	cholesterol	0.5
fish meal	48.4	astaxanthin	0.1
squid meal	10	vit. D3	0.01
yeast	1	vit. E	0.5
α -starch	18	taurine	0.5
fish oil	5	mineral	2
lecithin	1	vitamin mix	2
choline chloride	1		

Into the above ingredients steroids including 17 α -hydroxyprogesterone、Progesterone and 17 β -estradiol at a ratio of 1:6:2 of were added.

Results presented in Table 3 show that when experimental shrimps feeded with the immunostimulants inclueding β -Glucan from mushrooms, laminarin and sodium arginate, a significant upgrade of hemocytes in hemolyph was available comparing to the control shrimps. However, the best results was obtained when the shrimps was feeded with mushroom β -Glucan. The results also showed that during the course of experiment, β -Glucan treated shrimps showed the highest hemocyte counts. The presence of superoxide dimutase (SOD) may indicate the capability of shrimps in defence of existing stressors. The experiments performed on the detection of SOD in hepatopancrease through oral ingestion of β -Glucan lamiuarin and sodium aginate respectively were showed in Table 4. The results indicated that the feed supplemented with β -Glucan reveals the best SOD enhancing capability than those supplemented with laminarin and sodium aginate. Experiments performed on the field for cultured Pacific white shrimps (*L. vanmaemi*) feeded with mushroom β -Glucan is showed in Table 5. The results showed that cultured shrimp treated with β -Glucan revealing a very significant higher survival rates comparing with that of the control shrimp. However, due to high survival rates, cultured density maintained higher, the experimented shrimps showed a slightly slower growth than that of the control shrimps.

Table 3. Total haemocyte count in the haemolymph of Pacific white shrimp (*L. vannamei*) fed diets with different immunostimulants during the culture period.

Treatment	Dose	Days						
	(g/kg)	0D	1D	2D	4D	8D	16D	30F
Control		200.7±22.2	185.8±12.9^{c}	208.8±41.4^{e}	207.0±15.8^{d}	215.3±28.4^{d}	221.7±2.6^{d}	204.7±7.1^{e}
β-glucan	1		508.8±51.8^{a}	775.7±37.0^{a}	375.7±21.0^{b}	627.2±33.6^{a}	393.0±10.0^{a}	452.2±41.0^{b}
	4		517.8±49.0^{a}	550.8±54.1^{b}	469.0±26.9^{a}	431.8±77.0^{b}	376.7±13.1^{a}	502.5±23.4^{a}
Laminarin	1		509.0±64.7^{a}	549.3±64.2^{b}	309.8±33.6^{c}	240.0±50.6^{cd}	314.0±51.3^{b}	401.2±38.5^{c}
	4		373.7±21.1^{b}	303.5±8.2^{c}	226.2±27.5^{d}	383.0±20.9^{b}	282.7±7.7^{bc}	335.0±4.2^{d}
Sodium alginate	1		332.3±25.4^{b}	286.7±30.3^{cd}	308.0±10.6^{c}	300.5±14.1^{c}	363.3±27.8^{a}	327.8±8.5^{d}
	4		229.2±45.9^{c}	219.2±14.6^{e}	288.3±32.4^{c}	248.2±26.5^{cd}	263.0±32.5^{cd}	236.8±24.4^{e}

Values represent mean, and SD. a,b,c: mean in the same column with the different letter are significantly different (P<0.05).

Table 4. Superoxide dismutase (SOD) activity (U/mg protein) in the hepatopancrease of the white shrimp (*L. vannamei*) fed diets with different immunostimulants duing the culture period.

Treatment	Dose	Days						
	(g/kg)	0D	1D	2D	4D	8D	16D	30F
Control		0.51±0.12	0.56±0.14^{a}	0.43±0.03^{c}	0.51±0.05^{d}	0.45±0.14^{cd}	0.51±0.08^{a}	0.60±0.09^{bc}
β-glucan	1		0.62±0.13^{a}	0.91±0.13^{a}	1.65±0.29^{a}	0.76±0.19^{b}	0.54±0.13^{a}	0.27±0.04^{e}
	4		0.64±0.12^{a}	0.75±0.13^{ab}	0.89±0.11^{b}	0.48±0.03^{cd}	0.52±0.09^{a}	0.81±0.09^{a}
Laminarin	1		0.56±0.06^{a}	0.46±0.04^{c}	0.83±0.09^{bc}	0.58±0.08^{bc}	0.54±0.08^{a}	0.67±0.05^{ab}
	4		0.55±0.10^{a}	0.59±0.18^{bc}	0.82±0.11^{bc}	0.61±0.10^{bc}	0.28±0.06^{b}	0.58±0.16^{bc}
Sodium alginate	1		0.52±0.11^{a}	0.57±0.09^{bc}	0.50±0.11^{d}	1.10±0.19^{a}	0.55±0.19^{a}	0.34±0.6^{de}
	4		0.51±0.22^{a}	0.88±0.11^{a}	0.65±0.05^{cd}	0.30±0.04^{d}	0.56±0.12^{a}	0.48±0.08^{cd}

Values represent mean, and SD. a,b,c: mean in the same column with the different letter are significantly different (P<0.05).

Table 5. Surved and Growth of Pacific white shrimp (*Litopenaeus vannamei*).

		Initial culture density	Survival (%)	Harvesting Size
Experiment	1	35000/pond	30,000(85.71%)	12.05**±2.5**
Experiment	2	42000/pond	35,000(83.33%)	12.12**±3.2**
Experiment	3	38000/pond	29,000(76.32%)	12.53**±2.1**
Control	A	42000/pond	15,000(35.71%)	13.45**±1.7**
Control	B	45000/pond	13,000(28.89%)	13.82**±3.2**

Artifical feed containing 0.5% of β -Glucan were used for 1-3 Experiments.

DISCUSSION

White spot disease virus (WSSV) is demonstrated to be virulent to penaeid shrimps such as *Litopenaeus vannamei* and *Penaeus monodon* and considered to be responsible for serious economic losses on shrimp farms throughout the world. (Wongteerasupaya *at al.*, 1995; Lightner, 1998 and Lightner *et al.*, 2001) Many strategies for the prevention of disease occurrence were therefore performed (Acre *et al.*, 2000; Argue and Alcivar-Warren, 2000 and Bennie, 1998). Uses of specific pathogen resistant bloodstock or larvae was considered to be one of the feasible ways to prevent the occurrence of mass mortality due to pathogenic infections (Tang *et al.*, 2003). Studies performed by Argue *et al.*, (2002), Gjedrem (1997) and Gjedrem and Finland (1995) indicated that selective breeding of pacific white shrimp (*Litopenaeus vannamei*) either growth rate or resistance to virus infection may contribute to the successful shrimp aquaculture. Similarily, the studies performed in the present study showed that larvae derived from naturally selected white spot disease virus (WSDV) resistant broodstock exhibited significant resistance against WSDV. It was also showed that significant higher growth rate was obtained for the larvae of rapid growth shrimps. It is concluded that significant improvements on shrimp aquaculture can be made in Pacific white shrimp (*Litopenaeus vannamei*) through selective breeding in rapid growth and specific pathogen resistance. The results obtained from the present study also showed the uses of artificial feed with various steroid hormones, the maturation for Pacific White Shrimp may be significantly enhanced. This study may suggest that a complete cultivation for rapid growth and disease resistant *L. vannamei* can be accomplished for *L. vannamei*.

It is generally recognized that using of drug has been considered to be one of the most important negative impact factors for shrimp aquaculture. To resolve disease problem, shrimp aquaculture has therefore been foculized on strengthening defence mechanism by using immunostimulants. Amongst various immunostimulants, lipopolysaccharide and extract from *Fusarium* has demonstrated to be effective in the upgrade of total hemocyte count (THC) (Johansson *et al.*, 2000) for shrimps. Results obtained from Cheng *et al.* (2003) has demonstrated that feeding of β-1,3-Glucan may significantly enhance THC in viral infected shrimps. Similarily, the present study also showed that feeding of sodium alginate, laminarin and mushroom β -Glucan may significantly upgrading THC in the experimental shrimp. Amongst the above described materials, β -Glucan showed a best result on the enhancement of THC in experimental shrimps. Superoxid dismutase (SOD) is an active enzyme which may effectively eradicate the free-radicals in shrimp. The results obtained in the present study also showed that using of β-Glucan in feed may significantly and rapidly upgrading SOD activity in shrimp. The result may suggest that β-Glucan possesses a superior immunomodulating capability for shrimps. High survival rate obtained from field showed that using of β-Glucan in feed may significantly equip the shrimp with defence tool for shrimps. It is reasonable to anticipate that using of β-Glucan should be one of the most useful strategies for the prevention of viral or bacteria epizootics for cultured shrimps.

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Detection of *Vibrio parahaemolyticus* with a Chromogenic Medium

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ABSTRACT

Vibrio parahaemolyticus is a human pathogen that occurs naturally in the marine environment and fisheries products, particularly shellfish. The commonly used medium, thiosulfate-citrate-bile salts-sucrose agar (TCBS), for detecting this pathogen can not differentiate growth of *V. parahaemolyticus* from *Vibrio vulnificus* or *Vibrio mimicus*. Therefore, presumptive positive colonies formed on TCBS need to be confirmed with biochemical tests. This study evaluated a newly developed chromogenic medium, Bio-Chrome Vibrio medium (BCVM), for specific detection of *V. parahaemolyticus* based on formation of distinct purple colonies on the medium. When pure cultures of *V. parahaemolyticus* were tested with BCVM, all 148 strains grew with 145 of them produced purple colonies on the medium. Growth of *Vibrio vulnificus* or *Vibrio mimicus* on BCVM was easily identified by formation of blue green colonies while growth of *Salmonella, Shigella, Escherichia coli, Enterobacter cloacae, Yersinia enterocolitica,* and *Aeromonas* was totally inhibited. The BCVM was compared with TCBS for detecting *V. parahaemolyticus* in 296 seawater, sediment, and oyster samples. Higher false positive and false negative results were obtained by TCBS than BCVM. The accuracies of detecting *V. parahaemolyticus* were higher for BCVM (84%) than for TCBS (54%). BCVM was more specific and accurate than TCBS for *V. parahaemolyticus* detection.

Key words: *Vibrio parahaemolyticus*, Chromogenic medium, TCBS, Pathogen detection, Food safety.

INTRODUCTION

Vibrio parahaemolyticus is a seafood-borne pathogen that causes worldwide health problems associated with seafood consumption (Liston, 1990; Rippey, 1994; Kaysner and DePaola, 2001). This organism occurs naturally in coastal waters and is frequently found in molluscan shellfish, particularly oysters (Bej *et al.*, 1999; Wong *et al.*, 1999). Consumption of raw oysters contaminated with *V. parahaemolyticus* may lead to development of acute gastroenteritis characterized by diarrhea, vomiting and abdominal cramps. The infection may also cause life-threatening septicemia in persons having underlying medical conditions such as liver disease or immune disorders. To minimize the risks of *V. parahaemolyticus* infections associated with seafood consumption, effective post-harvest processes for eliminating *V. parahaemolyticus* in oysters and specific methods for detecting

V. parahaemolyticus need to be developed.

One of the commonly used methods for detecting *V. parahaemolyticus* in foods is the most probable number (MPN) procedure using thiosulfate-citrate-bile salts-sucrose agar (TCBS) (USFDA, 1998). However, this method is very labor-intensive and time-consuming because TCBS can not differentiate growth of *V. parahaemolyticus* from some strains of *Vibrio vulnificus* or *Vibrio mimicus*. Growth of *V. parahaemolyticus* as well as a few *V. vulnificus* and *V. mimicus* all appears as green or blue-green colonies on TCBS. Therefore, several presumptive positive colonies formed on a TCBS plate need to be confirmed by biochemical tests and the final results may not be available for 5 to 8 days.

Many methods, including molecular biology based polymerase chain reaction (PCR) and DNA hybridization (Nishibuchi *et al.*, 1985, 1986; Tada *et al.*, 1992; Yamamoto *et al.*, 1992, Bej *et al.*, 1999; McCarthy *et al.*, 1999), DNA probe combined with hydrophobic grid membrane filters (Banerjee *et al.*, 2002), and direct-plating procedures using non-radioactive DNA probes to detect the thermolabile hemolysin gene (*tl*) of *V. parahaemolyticus* (Gooch *et al.*, 2001), have been developed for rapid and specific detection of *V. parahaemolyticus*. However, these methods are much more complicate than the MPN method because of the involvement of DNA isolation, PCR primers preparation, DNA probes synthesis, and colony hybridization. In addition, special instruments and skilled technicians are essential for conducting the tests. Development of a simple and easy to use method for detecting *V. parahaemolyticus* would allow laboratory workers with no or limited microbiological training to perform the analyses.

Recently, a chromogenic medium (Bio-Chrome Vibrio medium) was developed for differentiating *V. parahaemolyticus* from other *Vibrio* species based on formation of distinct purple colonies by *V. parahaemolyticus* on the medium. Growth of other *Vibrio* species such as *V. vulnificus, V. mimicus,* and *V. cholerae* on the medium can easily be differentiated from growth of *V. parahaemolyticus* based on the formation of blue-green colonies. Although this newly developed chromogenic medium may provide an opportunity to reduce labor and time involved in *V. parahaemolyticus* detection, its application in detecting *V. parahaemolyticus* in foods and environmental samples needs to be validated. This study investigated the specificity and accuracy of this chromogenic medium for detecting *V. parahaemolyticus* in raw oysters and environmental samples.

MATERIALS AND METHODS

Bacterial cultures including 179 strains of Vibrio species (parahaemolyticus, vulnificus, cholerae, mimicus, hollisae, alginolyticus, fluvialis, and furnissii) and 42 strains of non-Vibrio bacteria, including Salmonella (choleraesuis, lansing, arizonae, havanna, enteritidis, oranienburg, stanley, houten, and meleagridis), Shigella (sonnei and flexneri), Aeromonas (hydrophila, veronii, sobria, and caviae), Enterobacter cloacae, Escherichia coli, Yersinia enterocolitica were used in this study.

Non-*Vibrio* bacteria were individually enriched twice in tryptic soy broth (TSB, Difco Laboratories, Detroit, Michigan, U.S.A.) at 37°C overnight and streaked onto both Bio-Chrome Vibrio medium (BCVM, BioMedix, Pomona, California, U.S.A.) and TCBS (Difco) plates for their abilities to grow on the media. Strains of *Vibrio* spp were individually enriched twice overnight in TSB supplemented with 1.5% NaCl at 37°C and streaked onto BCVM and TCBS plates to determine the specificity of BCVM for *V. parahaemolyticus* detection. All plates were incubated at 35-37°C for 18-24 hr and examined for growth and production of colored colonies.

A total of 296 samples (148 seawater, 74 oyster and 74 sediment) were collected from three oyster farms in States of Oregon and Washington. Freshly harvested oysters were briefly rinsed with estuarine water, shucked at the oyster plant, and placed in a plastic jar. Seawater and sediment samples were collected with sterile scoops and kept in sterile sampling containers. All samples were shipped in a cooler with ice to the OSU Seafood Lab. Presence of *V. parahaemolyticus* in samples were determined by the 3-tube MPN method according to procedures of United States Food and Drug Administration's Bacteriological Analytical Manual using both BCVM and TCBS plates. Presumptive positive colonies appeared on either TCBS or BCVM plates were confirmed as *V. parahaemolyticus* with MicroStation Microbial Identification System (BioLog, Hayward, CA, U.S.A.) according to manufacturer's instruction.

A multiplex polymerase chain reaction (PCR) targeting the *tl* (450 bp), *tdh* (269 bp) and *trh* (500 bp) genes in *V. parahaemolyticus* cells was used as a reference method for confirming *V. parahaemolyticus* in presumptive positive samples (Bej *et al.*, 1999; Kaysner and DePaola, 2001). The oligonucleotide primers for the *tl* (L-*tl*: 5'-aaa gcg gat tat gca gaa gca ctg-3' and R-*tl*: 5'-gct act ttc tag cat ttt ctc tgc-3'), the *tdh* (L-*tdh*: 5'-gta aag gtc tct gac ttt tgg ac-3' and R-*tdh*: 5'-tgg aat aga acc ttc atc ttc acc-3') and the *trh* (L-*trh*: 5'-ttg gct tcg ata ttt tca gta tct-3' and R-*trh*: 5'-cat aac aaa cat atg ccc att tcc g-3') genes were commercially synthesized (Integrated DNA Technologies, Coralville, IA, U.S.A.) according to the nucleotide sequences reported by Nishibuchi and Kaper (1985), Taniguchi *et al.* (1985) and Honda and Iida (1993),

Results obtained from the MPN method were compared with results obtained from PCR analysis. False-positive and false-negative results obtained by both BCVM and TCBS media were calculated based on PCR confirmed results. The accuracy of BCVM or TCBS for detecting *V. parahaemolyticus* was determined as 100 × [True Positive/(True Positive + False Positive + False Negative)]. A true positive result was defined as a positive result for *V. parahaemolyticus* that was confirmed by both PCR and the MicroStation System.

RESULTS

Growth of *Salmonella, Shigella, Enterobacter, Aeromonas, E. coli,* and *Yersinia* were completely inhibited by TCBS. Similar results were obtained by BCVM except for one strain of *Aeromonas hydrophila,* which grew and formed white colonies on the medium. All 148 strains of *V. parahaemolyticus* grew on BCVM and 145 of them produced purple colonies (Table 1). No purple colonies were produced on BCVM by other 31 *Vibrio* spp, except one strain of *V. fluvialis*. Growth of *V. cholerae, V. mimicus* and *V. vulnificus* on BCVM all produced blue-green colonies. Other *Vibrio* spp including *V. alginolyticus, V. furnissii,* and *V. hollisae* were either unable to grow or produced white colonies on BCVM.

Detection of *V. parahaemolyticus* in seawater, sediment and oyster with BCVM and TCBS is shown in Table 2. Among the 296

samples tested, 136 samples gave presumptive positive results on TCBS while 92 samples produced presumptive positive results on BCVM. The multiplex polymerase chain reaction (PCR) and biochemical tests (MicroStation System) confirmed that 77 samples (mainly seawater and sediment) were positive for *V. parahaemolyticus*. A total of 65 false results (62 false-positive and 3 false-negative) were observed for TCBS while only 15 (all false-positive) were reported for BCVM. The accuracies of TCBS and BCVM for detecting *V. parahaemolyticus* were determined as 54% and 84%, respectively (Table 3).

DISCUSSION

The selectivity of BCVM for detecting *Vibrio* spp was comparable to that of TCBS. Both media inhibited growth of many Gram-negative bacteria but allowed *Vibrio* bacteria to grow. However, BCVM was more specific than TCBS for *V. parahaemolyticus* detection. Growth of *V. vulnificus* and *V. mimicus* on TCBS that could not be distinguished from growth of *V. parahaemolyticus* could easily be identified on BCVM as blue-green colonies.

Table 1. Growth of *Vibrio* bacteria on Bio-Chrome Vibrio medium (BCVM) and thiosulfate-citrate-bile salts-sucrose agar (TCBS).

Vibrio spp.	No. of strains	BCVM	TCBS
V. alginolyticus	2	white	yellow
V. cholerae	13	blue green	yellow
V. fluvialis	1	purple	yellow
V. furnissii	1	white	yellow
V. hollisae	2	no growth	no growth
V. mimicus	2	blue green	green
V. parahaemolyticus	145	purple	green
	3	white	green
V. vulnificus	7	blue green	green
	2	no growth	green
	1	blue green	yellow

Table 2. Comparison of TCBS and BCVM with polymerase chain reaction (PCR) for detecting *V. parahaemolyticus*.

Test	Seawater	Sediment	Oyster	Total
TCBS (presumptive positive)	62	43	31	136
BCVM (presumptive positive)	41	35	16	92
PCR (positive)	32	33	12	77

Table 3. Accuracies of TCBS and BCVM for detecting *V. parahaemolyticus*.

Medium	Presumptive positive	False positive	False negative	Accuracy[a] (%)
TCBS	136	62	3	54
BCVM	92	15	0	84

[a] Accuracy (%) = [True Positive/(True Positive + False Positive + False Negative)] × 100. True positive = 77 (Confirmed by PCR and MicroStation System).

It is well known that TCBS may not differentiate growth of *V. parahaemolyticus* from some strains of *V. vulnificus* and *V. mimicus*. In this study, most of the bacteria producing false-positive results on TCBS were identified as *V. cholerae*, *V. mimicus*, or *V. vulnificus* (data not shown). However, none of the false-positive results obtained by BCVM were caused by any of these three species. *Vibrio vulnificus*, *V. cholerae*, and *V. mimicus* all appeared on the BCVM plate as blue-green colonies.

The formation of purple colonies by *V. parahaemolyticus* on BCVM seems to be a unique characteristic of the chromogenic medium. This study found that 98% of the 148 *V. parahaemolyticus* strains tested were able to produce purple colonies on BCVM. Although BCVM is not a perfect medium for detecting *V. parahaemolyticus*, it offers an advantage over TCBS for *V. parahaemolyticus* detection by being able to distinguish growth of *V. vulnificus* and *V. mimicus* from growth of *V. parahaemolyticus*. This is evidenced by lower false-positive and false-negative results obtained by BCVM than by TCBS. Since presumptive positive results obtained by TCBS needs to be confirmed with biochemical tests, using BCVM instead of TCBS resulted in a reduction of 44 confirmation tests (32% reduction). This result clearly indicates that BCVM can be used to reduce time and labor involved in *V. parahaemolyticus* detection.

Despite its higher specificity and accuracy than TCBS for *V. parahaemolyticus* detection, BCVM was not a perfect medium for detecting the organism. False-positive results were obtained from growth of some bacteria that were not *V. parahaemolyticus* (Table 3). Several bacteria including *Vibrio carcariae*, *Vibrio damsela*, *Vibrio fluvialis*, and *Enterococcus faecalis* were identified from samples that produced false-positive results on both TCBS and BCVM. However, there were a few bacterial isolates that appeared as presumptive positive colonies on both media but could not be identified by the MicroStation microbiological identification system. In addition, we were unable to identify the bacteria in 6 samples (3 seawater, 2 oyster and 1 sediment) that caused false-positive results only on BCVM. However, none of those unidentified bacteria were *V. parahaemolyticus* as determined by PCR analysis.

In addition to false-positive results, false-negative results were obtained for 3 samples by TCBS. All those 3 samples were found to contain very low levels of *V. parahaemolyticus* (<10 MPN/g), but were detected by BCVM and confirmed by PCR. It is believed that these false-negative results were obtained because the populations of *V. parahaemolyticus* in those samples accounted for only a very small portion of the total bacterial population. The green colonies produced by *V. parahaemolyticus* on TCBS were probably masked by a cluster of yellow colonies formed by other bacteria. However, the production of purple colonies on BCVM by *V. parahaemolyticus* in those samples was not affected by the large number of other bacteria. This result indicates that BCVM is more accurate in detecting low levels of *V. parahaemolyticus*.

It was interesting to observe that growth of *V. fluvialis* on BCVM also resulted in formation of purple colonies. It is not clear whether all strains of *V. fluvialis* will produce purple colonies on BCVM because only one strain of *V. fluvialis* was available for test in this study. Further investigation needs to be conducted to determine if all strains of *V.*

fluvialis would produce purple colonies on BCVM. Based on limited information obtained from this study, it appears that TCBS would be a better choice for *V. parahaemolyticus* detection if a large number of *V. fluvialis* is expected in samples. On the contrary, BCVM will work better than TCBS for *V. parahaemolyticus* detection when *V. vulnificus* or *V. mimicus* is expected in samples.

CONCLUSIONS

The Bio-Chrome Vibrio medium is more specific and accurate than TCBS for detecting *V. parahaemolyticus*. It can differentiate growth of *V. parahaemolyticus* from growth of *V. vulnificus* and *V. mimicus* and provides an opportunity to reduce time and labor involved in *V. parahaemolyticus* detection.

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Molecular Cloning and Characterization of Chitinase Gene *chiF* from *Streptomyces griseobrunneus* S3

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ABSTRACT

A preliminary survey of existence of family 19 chitinase gene among Taiwan native chitinolytic *Streptomyces* strains was conducted. Among 21 tested strains, 11 were detected positive by PCR (polymerase chain reaction) with the use of a family 19 chitinase gene specific primer pair. A full-length chitinase gene SGS3 *chiF* was cloned from *Streptomyces griseobrunneus* S3-a chitinolytic strain known to have great potential in plant disease control application. Sequence analysis revealed that the gene contains 891 bp which encodes a 296 amino acid peptide with a molecular weight around 31.5 KDa. The gene contains a chitin binding domain (ChtBD) with ChtBD consensus sequence AKWWTQ. On its upstream was a promoter region with characteristic direct repeat sequence (T/A) GGTC(T/C)AGACC(T/A) known to be critical in chitin induction and glucose repression. From a SGS3 *chiF* transformed *Escherichia coli* Rosetta™ (DE3), the chiF recombinant protein with a predicted size around 31.5 KDa was extracted and purified by Fast Performance Liquid Chromatography. The polyclonal antibody for immuno-detection of the recombinant protein was successfully prepared. The enzyme appeared to survive denaturing during SDS-PAGE analysis, the enzyme characteristics of recombinant protein was demonstrated by activity stain and western blotting. A phylogenetic analysis revealed that the cloned gene was highly homologous to the comparative gene known on *S. griseus*, *S. coelicolor*, and *Nocardiopsis prasina*, which might be adapted evolutionarily from the class IV chitinase of plant system. The molecular characteristics of the gene and its possible connection to antifungal characteristic of *Streptomyces* are herein discussed.

Key words: *chiF* gene, *Streptomyces griseobrunneus*, Family 19 chitinase.

INTRODUCTION

Chitin, an insoluble linear β-1,4-linked polymer of N-acetylglucosaminide (GlcNAc), is one of the most abundant polysaccharides in nature. It is a common constituent of insect exoskeleton, crustacean shell and fungal cell wall. The decomposition of chitin is important in the recycling of carbon and nitrogen resources in the biosphere. The presence of chitinase has been

implicated having critical importance physiologically and ecologically among bacteria, fungi, viruses, higher plants and animals. Chitinases known to date are classified into two categories- families 18 and 19, based on their polypeptide sequences and catalytic mechanisms (Armand *et al.*, 1994; Iseli *et al.*, 1996). The great majority of chitinases found to date are known to be members of family 18; whereas members of family 19 are unique in that they were found primarily in higher plants. *Streptomyces* are among micro-organisms the first known to possess family 19 chitinase (Ohno *et al.*, 1996; Saito *et al.*, 1999; Watanabe *et al.*, 1999).

In plant system, convincing evidences have demonstrated chitinases as one of the determinative factors contributing to active defence against the infection by plant-pathogenic microbes. The antifungal activity of class I, II and IV chitinases from various plant sources, especially those of family 19, has been well illustrated (Broekaert *et al.*, 1988; Iseli *et al.*, 1993; Leah *et al.*, 1991; Reborts and Selitrennikoff, 1988; Schlumbaum *et al.*, 1986). For family 19 chitinase recently discovered in *Streptomyces*, the role of the enzyme as an antifungal agent has been actively explored. A typical family 19 chitinase, chiC, discovered from *S. griseus* HUT6037 strain, was shown having anti-fungal activity on the agar plate against *Trichoderma reesei* (Watanabe *et al.*, 1999). And the *chiC* transgenic rice was shown having increased resistance against infection by *Magnaporthe grisea* (Itoh *et al.*, 2003).

In an attempt to explore microbial resources for biopesticide development, *Streptomyces griseobrunneus* S3 (SGS3) was selected from cohorts of rhizosphere microbes for its superior chitinolytic activity and antagonistic effect against phytopathogenic fungi. By a dual culture assay, the bacterium was found strongly antagonistic against *Pythium aphanidermatum*, *Phytophthora parasitica*, *Colletotrichum gloeosporioides*, *Guignardia citricarpa*, *Pyricularia oryzae* and *Rhizoctonia solani* AG4 (Lai, 2003). It's potential as a biofungicide for plant disease control has been demonstrated, and the disease control efficacy was shown due at least in part to the mycoparasitic activity. The functioning of chitinolytic activity appeared to be critical in the anti-fungal activity observed. In order to elucidate the molecular basis of the biocontrol efficacy, the possible involvement of chitinase genes of this bacterium was investigated. A family 19 chitinase gene *chiF* was shown in an *in vitro* culture system among the surveyed chitinase genes preferentially expressed when colloidal chitin was applied. The full-length SGS3 *chiF* was cloned, and the characteristics of the gene expression and the enzyme cleavage activity were further investigated. Also, the existence of *chiF* gene among various chitinolytic antagonistic bacterial/fungal strains was surveyed. The molecular and biochemical characteristics of the gene and the encoded enzyme protein are herein discussed.

MATERIALS AND METHODS

Microbial strains culturing and preservation

A total of 21 *Streptomyces* strains shown in Table 1 were used for this investigation. All the test strains had been shown to have superior chitinolytic characteristics in our previous works (Lai, 2003). Except S16 and SS31 which were kindly provided by Prof. Jenn-Wen Huang and Dr. Tung-Tsuan Tsai, each respectively, all other tested strains were isolated from rhizosphere in a serial

screening works aiming for chitinolytic antagonists with potential in plant protection application.

All the bacterial strains were cultured on potato sucrose agar (PSA) plate at 30℃ in dark for 3 days. The bacterial spores/ mycelia were then harvested and suspended in sterilized distilled water containing 0.05% (v/v) Tween 20 and stored at 4℃ for long term preservation. The tested fungal strains were cultured on PSA plate at 30℃ for 5 days, and then stored at 4℃. For experiments conducted, the microbes were allowed to grow on PSA plate as above indicated. And the freshly prepared spores/mycelia suspensions were then used as inoculum for the experiments.

Extraction of total DNA from *Streptomyces*

Genomic DNAs were extracted from *Streptomyces* species by the method described by Kutchma et al. (1998). The test bacteria were cultured in 20 ml potato sucrose broth in 250 ml flask under continuous shaking at 120 rpm. The mycelia were collected by 10 000×*g* (SS-34 rotor, Sorvall® RC5C) centrifugation at 4 ℃ from overnight culture, suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7) containing 1 mg/ml lysozyme (Amresco Chemical) and incubated at 37℃ for 1-2 h or until the suspension turn slightly viscous due to partial degradation of cell wall. Sodium dodecyl sulfate (SDS) and NaCl were then added to final concentration at 1% and 1 M, respectively, and the sample was then subjected to repeated freeze/thawing for 3 times. After removing cell debris by centrifugation, the DNAs were purified by phenol/chloroform extraction and ethanol precipitation. The genomic DNAs obtained were then used for PCR and southern analysis.

Table 1. *Streptomyces* strains used in this study.

Strains	Sources
S1 (*Streptomyces* sp.)	Peat moss, Soil Mix, HEVECO, Canada.
S2 (*Streptomyces* sp.)	Peat moss, Floral Guard, TKS, Germany.
SGS3 (*S. griseobrunneus*)	Citrus rhizosphere, Taiping, Taichung County.
S4 (*Streptomyces* sp.)	Peat moss, Soil Mix, HEVECO, Canada.
S5 (*Streptomyces* sp.)	Taro rhizosphere, Dali, Taichung County.
SL7-1 (*S. lincolnensis*)	Sesbania rhizosphere, Hsingkang, Chayi County.
SL7-2 (*S. lincolnensis*)	Sesbania rhizosphere, Hsingkang, Chayi County.
S8 (*Streptomyces* sp.)	Taiwan Anoectochilus rhizosphere, Taichung.
S9 (*S. fimbriatus*)	Sugarcane rhizosphere, Hsingkang, Chayi County.
S10 (*Streptomyces* sp.)	Grape rhizosphere, NCHU, Taichung.
S12 (*Streptomyces* sp.)	Sesbania rhizosphere, Hsingkang, Chayi County.
S14 (*Streptomyces* sp.)	Sugarcane rhizosphere, Hsingkang, Chayi County.
S15 (*Streptomyces* sp.)	Soil, Datsun, Changhwa County.
S16 (*Streptomyces* sp.)	Mushroom growth substrate, Sinshe, Taichung County.
S18 (*Streptomyces* sp.)	Sugarcane rhizosphere, Hsingkang, Chayi County.
S20 (*Streptomyces* sp.)	Citrus rhizosphere, Taiping, Taichung County.
SS31 (*S. saraceticus*)	Dead cicada remain in citrus orchard.
S38 (*Streptomyces* sp.)	Peanut rhizosphere, Taiping, Taichung County.
SC15 (*Streptomyces* sp.)	Soil, Wufeng, Taichung County.
SC17 (*Streptomyces* sp.)	Soil, Wufeng, Taichung County.
SC19 (*Streptomyces* sp.)	Soil, Wufeng, Taichung County.

† Strain S31and S16 were each respectively kindly provided by Prof. Jenn-Wen Huang and Dr. Tung-Tsuan Tsai, Department of Plant Pathology, National Chung Hsing University, Taichung, Taiwan.

Molecular cloning and detection of the expression of partial *chiF* from SGS3

Partial sequence of *chiF* was amplified by PCR extension by use of specific primer pair shown below:

ChiFF	5′AAG CGC GAG GCC GCG GCC
ChiFR	5′GCA CTC CAG AGC GCC GTT GAT 3′

The primers designed for *chiF* was based on the nucleotide sequence of *chiC* (accession number AB009289) of *S. griseus* (Watanabe *et al.*, 1999) in corresponding to the amino acid sequences from Lys-134 to Ala-142 and from Ile-256 to Cys-262, respectively. With the addition of SGS3 genomic DNA as template, PCR amplification was performed for 30 cycles by the scheme at 94℃ denaturation for 30 sec, 62℃ reannealing for 30 sec, and 72℃ extension for 30 sec; and a final cycle at 72℃ for 5 min. To avoid the formation of DNA secondary structure due to high GC content of *Streptomyces* genome, 1% dimethyl sulfoxide (DMSO) and 1 M betaine were amended in PCR reaction buffer (Baskaran *et al.* 1996). The amplicons were then cloned with TA-cloning kit (yT&A TA cloning kit, Yeastern Biotech Co., Taipei, Taiwan). For southern and northern analysis, digoxigenin (DIG) was incorporated into these cloned sequences according to the manufacturer's protocol (DIG DNA labeling kit, Roche, Mannheim, Germany).

Preparation of colloidal chitin and glycol chitin

Colloidal chitin was prepared by the method described by Lingappa and Lockwood (1962) using powdered crab shell chitin purchased from Sigma (St. Louis, MO, USA). The chitin powder was digested with 50% sulfuric acid for 10 min, washed with distilled water several times until pH reached 6-7, and then diluted to 2% (w/v) with distilled water for medium preparation. Glycol chitin was prepared by the method described by Yamaga and Imoto (1981). Approximately 1 g of glycol chitosan (Sigma, St. Louis, MO, USA) was immersed in 20 ml of 10% acetic acid overnight, washed with 90 ml methanol, reacted with 1.5 ml of acetate anhydride under continuous stirring at room temperature for 30 min, and then washed again with methanol for several times. The glycol chitin thus obtained was then precipitated by centrifugation at 5000 × *g* (SS-34 rotor, Sorvall® RC5C), suspended in 0.02% (w/v) sodium azide and stored at 4℃ until use.

Detection of *chiF* among tested *Streptomyces* strains

Genomic DNAs of all 21 tested *Streptomyces* strains (listed in Table 1) were extracted by the method described above; the existence of *chiF* was detected by PCR amplification using the primer pair *chiF*F/*chiF*R. The PCR amplification scheme was same as that above described.

Cloning of *chiF* from SGS3

The genomic library of SGS3 was constructed by use of ZAP Express® Predigested Vector Kit and ZAP Express® Predigested Gigapack Cloning Kits (Stratagene, CA, USA). Genomic DNA was restriction digested with *Bam*HI and resolved by agarose gel electrophoresis. The resolved fragments near 3 kb were recovered and ligated to the ZAP express vector according to the manufacturer's protocol. The *chiF* recombinants were screened by

southern analysis using the DIG-labeled partial *chiF* fragment as detection probe, the contained insert was then fully sequenced.

Construction of *chiF* expression vector and purification of the recombinant protein

For construction of the expression vector, the coding region of *chiF* was amplified by PCR with the use of a forward primer (5′-GATCCATGGTGTTCAAACGTGTCCTC-3′) which contains sequence identical to N-terminal of a *Nco*I site (sequence underlined) inserted *chiF* gene flanking at its 5′ end, and a reverse primer (5′-GATGAATTCCCGCAGCTCAGGTTCG A-3′) which contains sequence complementary to the C-terminal of an *Eco*RI site (sequence underlined) inserted *chiF* flanking at its 5′ end. As that shown in Fig. 1., the amplicon was then ligated to a *Nco*I- and *Eco*RI- digested plasmid vector pET26b (Novagen, EMB Biosciences Inc., Germany) following the manufacturer's protocol.

The constructed plasmids were then used to transform competent cells of *E. coli* Rosetta™ (DE3), and the recombinant strains which harbor the expression plasmid pET26b/*chiF* were selected and allowed to grow at 37℃ in LB medium containing kanamycin (100 μg/mL). The suspended cell cultures were fed with 1 mM of IPTG when the A_{600} of the cell suspension reached 1.0, the cells were then allowed to grow for another 4 hr at 28℃. The cells were then pelleted by centrifugation (10 000 × *g*, GSA rotor, Sorvall® RC5C), and the recombinant chiF protein which exist predominantly in periplasmic fraction was extracted by an osmotic shock facilitated protocol (manufacturer provided) and concentrated by ammonium sulfate precipitation. The recombinant proteins obtained were dissolved in 20 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl and resolved by a ÄKTA FPLC system with the use of Superdex 200 HR 10/30 column (Amersham Biosciences, Cardiff, UK). The samples were eluted with the same buffer at 0.5 ml/min flow rate, and desalted by an Amicon® Ultra-15 centrifugal filter device (Millipore, Billerica, Mass.,USA) where 20 mM phosphate buffer (pH 7.0) was applied as equilibrating buffer.

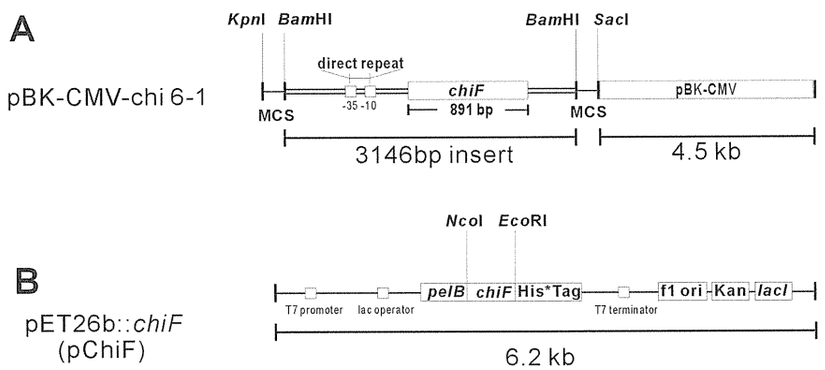

Fig. 1. Construction scheme of cloning vector pBK-CMV-chi 6-1(A) and SGS3 *chiF* overexpression vector pChiF (B).

Detection of chitinase activity in SDS-PAGE with glyco chitin amendment

SDS-PAGE was conducted as that described by Bollag *et al*. (1996) except that the acrylamide gel was amended with 0.01% glycol chitin. The electrophoresis resolved gel was washed with renaturing buffer [consists of 0.1 M sodium acetate, 1% (w/v) triton X-100, pH 5.0] for 20 min twice and then stained with 0.01% (w/v) Calcofluor White M2R (Sigma, St. Louis, MO, USA) (dissolved in 0.5 M Tris-HCl, pH 8.9). It was then destained with distilled water and the chitinolytic band was visualized with UV light.

Preparation of chiF polyclonal antiserum

The recombinant chiF protein obtained from ÄKTA FPLC purification was used to immunize New Zealand white rabbit. The protein samples were adjusted to 2.0 mg/L in concentration by the addition of 20 mM sodium acetate buffer (pH 5.8). Approximately 1 mg of the sample was injected on a weekly basis. The first injection was applied with the addition of equal volume of Freund's complete adjuvant (Difco Laboratories Detroit, Michigan), whereas the followed three injections were done with the addition of Freund's incomplete adjuvant. The rabbit was bled 1 week after the last injection. The antiserum obtained was dispensed in eppendorf tubes and stored at -20℃ for further application.

Detection of chiF by western blotting

The chiF protein resolved by SDS-PAGE was electro trans-blotted onto nitrocellulose membrane by use of BioRad transblot apparatus (Bio-Rad, Hercules, CA). The western blot analysis was performed by the protocol described by Sambrook *et al*., (1982) using chiF polyclonal antibody prepared.

Phylogenetic analysis of *chiF* clonned

The 145-257th amino acid sequence (113 amino acids in length) as deduced from the catalytic domain of *chiF* was used for the analysis performed. The comparative amino acid sequences- with the lengths ranging from 102-137 amino acids, which were available on gene bank of NABI known from a total of 29 different biological origins (Table 2) were used for the analysis.

The analysis was conducted with the use of DNASTAR sequence analysis software (DNASTAR, Madison, USA). The amino acid sequences alignment was processed by MegAlin program of DNASTAR by the method of Clustal W.; the neighbor- joining method was used for the sequence comparison and the construction of the phylogenetic tree.

RESULTS

Molecular cloning and detection of *chiF* expression in SGS3

With the application of the designed primer pair and the use of SGS3 genomic DNA as template, the partial sequence of *chiF* was amplified and cloned. The size of cloned *chiF* was 387 bp. The cloned partial gene in accordance with each respective corresponding chitinase gene on *S. griseus* and *S. coelicolor* were illustrated on Fig. 2.

Detection of *chiF* among tested *Streptomyces* strains

The PCR amplification using *chiF*F /*chiF*R as primer pair indicated that eleven test strains including S1, SGS3, S4, S5, SL7-1, SL7-2, S9, S10, S12, S14, and S38 were detected positively with *chiF* on their

genomic DNAs (Fig. 3). The rest 10 test strains, however, showed negative responses in the PCR amplification conducted.

Table 2. Chitinase peptide sequences used for comparison in this study.

Accession Nos.	Source organisms	Protein ID.
AAA56787	Barley	chitinase, class I
AAA51377	Rice	chitinase, class I
AAA32769	Thale cress	chitinase, class I
CAA87072	European elder	chitinase, class I
CAA33517	Potato	chitinase, class I
CAA34812	Tobacco	chitinase, class I
CAA39535	Rice	chitinase, class I
AAA34107	Tobacco	chitinase, class II
CAA47921	Potato	chitinase, class II
CAA35791	Petunia	chitinase, class II
AAA32941	Barley	chitinase, class II
CAA40474	Kidney bean	chitinase, class IV
AAA32916	Beet	chitinase, class IV
CAA43708	Rape	chitinase, class IV
AAA33444	Maize	chitinase, class IV
BAA84194	*S. coelicolor*	Partial family 19 chitinase
BAA84195	*S. lividans*	Partial family 19 chitinase, f1
BAA84196	*S. lividans*	Partial family 19 chitinase, f2
BAA84197	*S. lividans*	Partial family 19 chitinase, f3
BAA84199	*S. coelescens*	Partial family 19 chitinase
BAA84200	*S. ipomoeae*	Partial family 19 chitinase, f1
BAA84201	*S. prasinopilosus*	Partial family 19 chitinase, f1
BAA84202	*S. prasinopilosus*	Partial family 19 chitinase, f2
BAA84203	*Streptomyces* sp. Strain S15	Partial family 19 chitinase
BAA84204	*Streptomyces* sp. Strain S84	Partial family 19 chitinase
BAA84205	*Streptomyces* sp. Strain S100	Partial family 19 chitinase
BAA84206	*Streptomyces* sp. Strain S159	Partial family 19 chitinase
BAA23739	*S. griseus*	Complete family 19 chitinase,
AY953370	*S. lincolnensis* (SLS7)	Partial family 19 chitinase

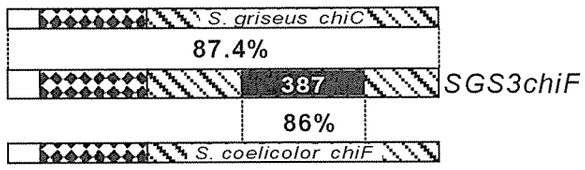

Fig. 2. An illustration (modified from Saito et al. 2000) showing sequence identity of the cloned SGS3 *chiF* comparing to each of the corresponding genes known in *S. griseus* and *S. coelicolor*. The black box indicated the partial *chiF* cloned, the size (bp) was printed in white on black box. Numbers between the regions of the gene show the percentage of sequence identity with compared gene fragment. Open boxes, signal sequence; checkered boxes, substrate-binding domain; and striped boxes, catalytic domain.

The detection by northern analysis of chitinase gene expression in SGS3 grown in colloidal chitin (0.01%) amended Czapek Dox Broth indicated that the expression of *chiF* appeared to be transient which reached a maximum level generally 2-4 h after addition of colloidal chitin and decreased thereafter (data not shown). The expression *chiF* was greatly repressed, however, without colloidal chitin amendment or when glucose was added as the only or the additional carbon source (data not shown). An adjunct Blast analysis indicated that *chiF* belongs to the member of family 19 chitinase, which has been implicated to be critical in chitin degradation and antifungal activity of members of *Streptomyces* (Watanabe *et al.*, 1999).

Cloning of full-length *chiF* gene from SGS3

From the genomic library constructed, a total of 42 *chiF* positive phage clones were selected by southern analysis. Among them, clone chi 6-1 that harbors an insert of 3146 bp (Fig. 4) was arbitrarily selected and fully sequenced.

The computer analysis with Seq Web Version 2 software (adapted from http://apdb1.nhri.org.tw:8003/gcg-bin/seq web.cgi, Accelrys, Inc.) indicated that the insert contains only one open reading frame of 891 bp in size (Fig. 5). The gene contains a secretory signal peptide domain, a chitin binding domain (ChtBD) and a catalytic domain. The open reading frame encoded a polypeptide of 296 amino acid residues and a predicted polypeptide at molecular weight of 31.5 kDa. A ChtBD consensus sequence AKWWTQ (Tsujibo *et al.*, 2003) was found at the 24-29th amino acids of the deduced peptide sequence. A possible promoter sequence TTGAGT (-10, single underlined) and TTGACA (-35, double underlined) were found at 62 and 86 upstream of the translation site (Figs. 5 and 6). And in adjacent to each of them was the direct repeat sequence TGGTCTAGACCT and AGGTCCAGACCA (Delic et al. 1992; Ni and Westpheling 1997). The cloned *chiF* gene was 87.4% identical to *S. griseus chiC*. The resolved *chiF* nucleotide sequence has been submitted for registration by National Center for Biotechnology Information and is now available by the accession number AY348315.

Production and purification of SGS3 chiF from *E. coli*

From broth culture of *E. coli* Rosetta™ (DE3) that harbors the transformed pET26b/*chiF*, production of chiF protein was achieved by IPTG induction. The chiF protein extracted was concentrated by ammonium sulfate (at 90% saturation) precipitation, resuspended with phosphate buffer (20 mM, pH 7), and resolved by chelating gel on an ÄKTA FPLC system. A protein peak (arrow indicated) with expected enzymatic activity was detected (Fig. 7).

A SDS-PAGE analysis indicated the recombinant protein has molecular weight around 31.5 kDa (Fig. 8A lanes 1 and 2). The followed western blot analysis further revealed that the detected protein band reacted positively with chiF antibody prepared (Fig. 8B). The chitinolytic activity of the major band was demonstrated by fluorescent staining with Calcofluor white M2R (Fig. 8C) and as well by development of clear chitinolytic zone on the colloidal chitin amended agar plate (Fig. 8D).

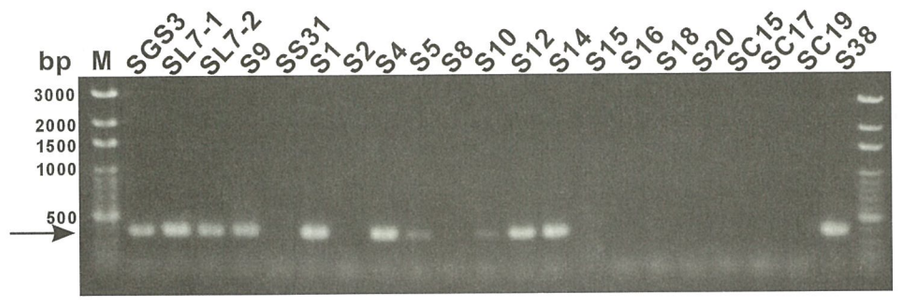

Fig. 3. PCR amplification of family 19 chitinase from 21 *Streptomyces* spp. strains by using primer pair *chi*FF/*chi*FR. The arrow indicated the predicted 387 bp amplicon of the partial family 19 chitinase gene.

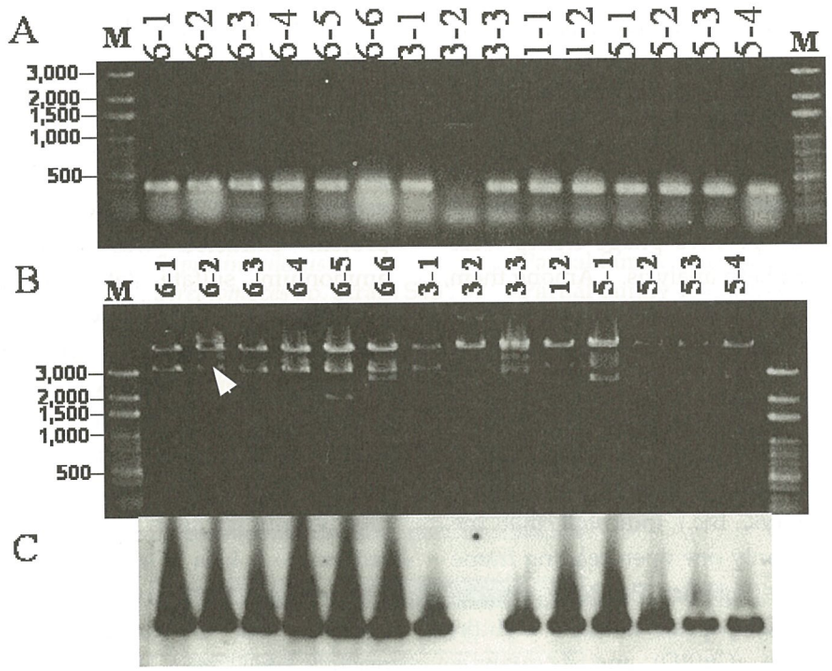

Fig. 4. Detection of *ChiF* gene among the transformed *E. coli* isolates obtained. The *ChiF* gene was detected through PCR amplification using primer set ChiFF/ChiFR(A). The phagemid DNAs from each isolates were digested with *Bam*HI and resolved by 1% agarose(B) and subsequently hybridized with *ChiF* probe (C). The arrow designated the target 3kb DNA fragment containing *ChiF* gene. Lane M, 100 bp DNA ladder marker (GeneMark).


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1    ggatccggtc cccgccgagg ttctccgcga ggtggtacga ggcccgtgtg ccgggcaccg gcaccacgtg gtcaccgagc cggagcagcc atgccagggc
101  gacctgcgcg ggggtggcct cgtgccgggc cgccacctgg gcgacggcct gcgtgagcgc gtagttgtgg cggatggccc gcgggggcgaa gcgcggctgg
201  gtgcgccgcc agtcgtccgg ggccaggtcc tcggtgcggg agtacgcgcc ggtcagcagc ccgcgcccga gcggggcacc ggccagcagg gcgatccggc
301  gctccaccgc gtacggcacg acgtcgtcga ggccgcaccg cgtgaacagc gacacctcgg cgccgatcgc cgccgcctcg tgcacggcct cggcctcgcg
401  ggcctgctcc acggtgacga cgggcagccc cagcatgcgg atcttgcccg cccagacctg ctcggccagc gccccccagc tctccgtcag cggtacggcg
501  gggtcgatgc ggcgcagcag atagaggtcg atgtggtcca cgtgcagcct gcgcaggctc tcgtcgaccg cgtcccgcag cgcctgcgga ccgcccgcgg
601  gccgcagcac cccgtccggg ttgccgagca ggccgccgcc ggtgcagagc accacctcgt cccgccgccc ccggatggcc tcaccgatca gccgctcgtt
701  ctccccgtcc gcgtaggcgt cggccgtctc cagcaggttg accccgaggt cgaccgcctc ctggacgagc gacaccgcct tggtccggtc caccaggccg
801  ggcgcgtagc ggtacgagag acccatcgcg cccagtccca gccggccgac gaccggtccg tccctgccca tcgttgtggt ccgcatccgg ggcgaacctc
901  cttctccaca gaggaatgtg acccccctcat ggcagcagcc ccggcagccg gagcacagca ttccgctcct acttgccgac ctccgacgac ttcgggaagt
1001 ccgacggcgt tccgggggcgg gaagtccgac gtgtcccggg ccgggaggcc ccgaggcgtt ccggtccggg aagtccgacg acgcaccggt cccgggaggc
1101 ccgaaggtgt tccggtccgg gaagtccgcc ggtgtcccgc atcctggacg gtccgggaat ttttacctcc ggtttccgcc ctccccgtac cgcgatgtga
1201 aagaactccg accggaatca ggccagttga gaggtgggat ggcatcgtta tcaggaggcg acggcggccc gtcgcgacac gtgcgccgac gtcacgaagg
1301 atggtctaga ccttgacagg tccagaccat catcgcttga gtgtcggcac ccccacggcg gcgcacggcc ggacgtagcg ccgcgcctaa ggagtgtgtt
1401 cac gtg ttc aaa cgt gtc ctc agc ctg ctg gtc gcg ctc ggc gcg gtc gtc gcg gcg ttc gtc gtc ctc ccc gcc gcc acc gcg
        M  F  K  R  V  L  S  L  L  V  A  L  G  A  V  V  A  A  F  V  V  L  P  A  A  T  A
1485 cag gcc gcc acc tgc ttc agc gcc tgg aac tcc tcg tcc gtc tac acc ggc ggc cag acc gcc tcg tac aac ggc cgg aac tac
     Q  A  A  T  C  F  S  A  W  N  S  S  S  V  Y  T  G  G  Q  T  A  S  Y  N  G  R  N  Y
1569 acc gcc aag tgg tgg acc cag aac gag cgt ccg ggc tcc tcc gac gtc tgg acc gac aac ggc gcc tgc ggc acg ggc ggc ggc
     T  A  K  W  W  T  Q  N  E  R  P  G  S  S  D  V  W  T  D  N  G  A  C  G  T  G  G  G
1653 ggt gag gac ccg ggc cag agc aac ggg ttc gtc gtc agc gag gcg cag ttc aac cag atg ttc ccg aac cgg aac tcg ttc tac
     G  E  D  P  G  Q  S  N  G  F  V  V  S  E  A  Q  F  N  Q  M  F  P  N  R  N  S  F  Y
1737 acg tac cgg ggt ctc acc gac gcc ctg agc gcg tac ccc gcc ttc gcc aac acc ggc agc gac gag atc aag aag cgc gag gcc
     T  Y  R  G  L  T  D  A  L  S  A  Y  P  A  F  A  N  T  G  S  D  E  I  K  K  R  E  A
1821 gcg gcc ttc ctc gcc aac gtc agc cac gag acc ggc ggg ctg gtg tac atc gag gaa gtc aac gag gcc aac tac ccc cac tac
     A  A  F  L  A  N  V  S  H  E  T  G  G  L  V  Y  I  E  E  V  N  E  A  N  Y  P  H  Y
1905 tgc gac gcc agc cag ccc tac ggc tgc ccc gcc ggc cag tcc gcg tac tac ggc aag ggc ccg atc cag ctg agc tgg aac ttc
     C  D  A  S  Q  P  Y  G  C  P  A  G  Q  S  A  Y  Y  G  K  G  P  I  Q  L  S  W  N  F
1989 aac tac aag gcc gcc ggt gac gcg ctc ggc atc gac ctg ctg aac aac ccc tac ctg gtc gag cag aac gcc gcc gtc gcc tgg
     N  Y  K  A  A  G  D  A  L  G  I  D  L  L  N  N  P  Y  L  V  E  Q  N  A  A  V  A  W
2073 aag acc gcc ctc tgg tac tgg aac acc cag acc ggc ccc ggc acc atc acg ggc cac aac gcc atc gtc aac ggc ccc ggc ttc
     K  T  A  L  W  Y  W  N  T  Q  T  G  P  G  T  I  T  G  H  N  A  I  V  N  G  P  G  F
2157 ggc gag acg atc cgc tcc atc aac ggc gcg atc gag tgc aac ggc ggc aac ccg gcc cag gtc cag agc cgc atc gac aag ttc
     G  E  T  I  R  S  I  N  G  A  I  E  C  N  G  G  N  P  A  Q  V  Q  S  R  I  D  K  F
2241 acg cag ttc acg cag gtt ctc ggc acc acg acc ggg tcg aac ctg agc tgc tga ttccgg gcgcgttcca cgaacgtcga acgttccccg
     T  Q  F  T  Q  V  L  G  T  T  T  G  S  N  L  S  C  *
2331 cgcgacccgt cccgtacggg ggtccgcccg gctgctgccg ggagggcccc cgttccggcg ttccggccgt tccggcgttc cgggagacgg tcggcaggat
2431 cgggtacgtc aggccagcgg cggcttcacc gacatcagca gatgccggtg gcggggaggg gagacgtccc ggccgggttc accctcgccc gtgatcatcg
2531 cccgctgccc cgcccccatc atcagcagcc gcagcgacgg ctcgtcgaag gaggccaggg cgtccgccag atacgccggg ttgaacgcca cggtcatctc
2631 gcgggcgcct tccagggtcg cgggcaaccg ctgggaggcc acgtcgtcct cgtaaccggc tgcagatgta cggagttgtc gtcggcggag aacgccaatt
2731 ggaccggccc gtcccgtcgg cgaccaccga gacccgcttg acggcctcca ccagccgcgc ccggtccacc accgccactg ccgggtcctc catcgcgaag
2831 agcttgttgt ggcgcgggag ccgtccgtcg agcagtcgca cggtggtccg catcccgcgg tgctcgaacc ccgccgaacc ggcgcccagg gcgacgctca
2931 ccagcccgga ccgccccagc gaacgggcga tctcggtcaa ccgccgcgcg gagaccagca catcggcact ttcaccggcc tcggaccctt cacccgcccc
3031 cgtatccgca cccgccccgg ccgccttcca aggcaacgtc cgtacggcga accggtaccg gtcggtcgcg gcgagcgtca tcgtccccgt cccgcgtcg
3131 agccccagcc ggatcc
```

Fig. 5. Nucleotide sequence and deduced amino acid sequence of SGS3 *ChiF* gene of *S. griseobrunneus* S3. Amino acids are specified in single-letter code under the first of each codon. The -35 and -10 sequence of each promoter are double- and single-underlined, respectively. Shadow box and open box indicated the secretory signal peptide and chitin binding domain (ChtBD), respectively. Catalytic domain is printed in white on black boxes. It consists 891 bp in length, which encodes a 296 amino acid peptide with a molecular weight of 31.5 KDa. A ChtBD consensus sequence AKWWTQ (shown in bold type) was found in the gene at 24-29th amino acids of the peptide sequence.

p-*chiF* gaacgcaagttgaacTGGTCTACACCCttgacTGGTACAGACCAaagcggttgagtatgcctccacacc

SGS3 *ChiF* ccgacgtcacgaaggaTGGTCTAGACCTtgacAGGTCCAGACCAtcatcgcttgagtgtcggcaccccc

Consensus TGGTC(C/T)(A/G)GACC(A/T)

Fig. 6. Nucleotide sequence of the promoter regions of *chiF* gene (p-*chiF*) of *S. coelicolor* A3(2) and *ChiF* gene of *S. griesobrunneus* S3. The –35 and –10 sequence of each promoter are double- and single-underlined, respectively. Nucleotide sequences of direct repeats are printed in upper-case letters and those identical to the consensus sequence (Saito *et al.* 2000) are printed in white on black background.

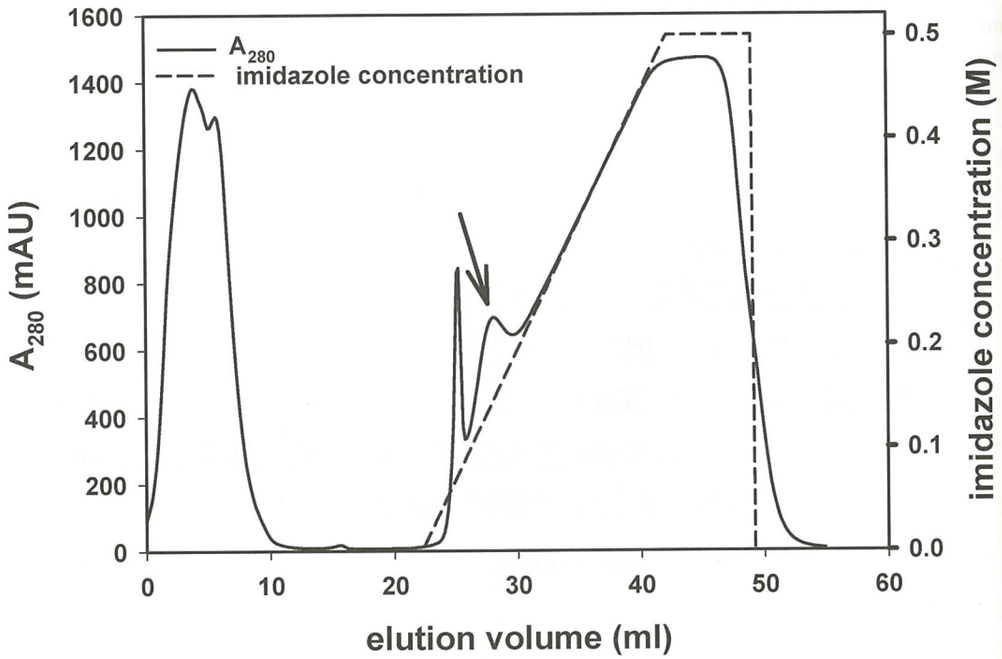

Fig. 7. Purification of chiF protein by chelating gel on an ÄKTA FPLC system. Ammonium sulfate precipitated SGS3 chiF protein was dissolved in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4) and applied onto HiTrap Chelating HP column. The arrow indicates SGS3 chiF protein peak eluted.

Phylogenetic correlation of *chiF* among the known Family 19 chitinase genes

The pileup alignment of the catalytic domain family 19 chitinase amino acid sequences known from 15 *Streptomyces* strains comparing to that from 15 plant resources indicated clearly the existence of high sequence identity among the *Streptomyces* strains analyzed (Fig. 9). The high sequence identity was shown further by the phylogenetic tree analysis where that the 15 test *Streptomyces* derived chitinases were sorted into one group which appeared to be distinct from all the compared plant derived sequences (Fig. 10). The alignment analysis also indicates the close relatedness among these *Streptomyces* enzymes and the compared plant derived enzymes, the identical sequences was detected mainly from the region ranging from the 60-110th amino acids (Fig. 9). What worth noting was that these enzymes appeared to share high similarity to all the plant Class IV chitinase tested including that from European elder, kidney bean, rape, beet and maize, in that there were deletion sequences at 33-50th and 111-131st amino acid sequence region. In contrast to this, the deletion regions were not detected among the plant derived Class I and Class II enzymes tested (Fig. 9).

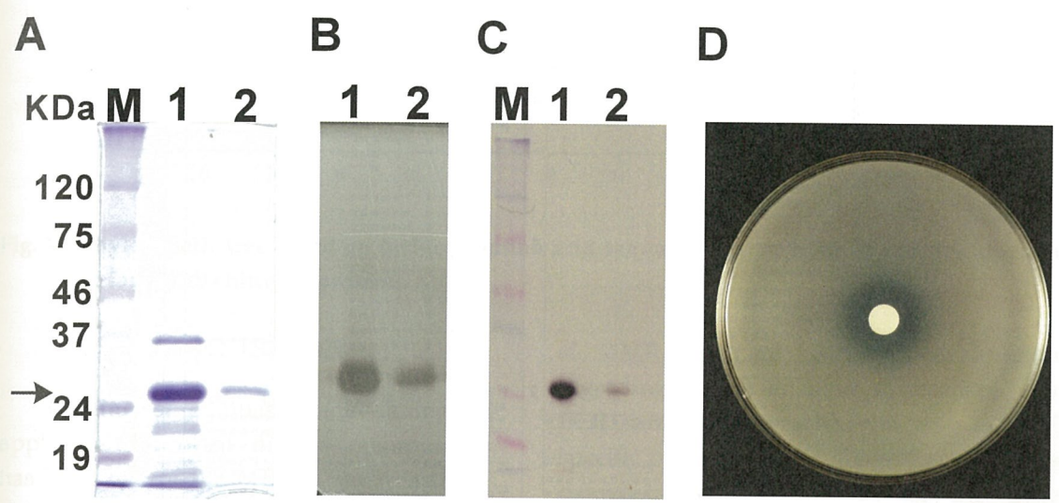

Fig. 8. Detection of recombinant SGS3 chiF protein by SDS-PAGE (A and B), western blot (C), and by clear zone formation on 0.5% colloidal chitin amended agar plate (D). Recombinant chiF was extracted by ammonium sulfate precipitation and purified by chelating gel chromatography. For SDS-PAGE analysis, approximately 10 μg of the protein sample were loaded on each lane, and the resolved gel was stained with Coomassie blue (A) or with Calcofluor white M2R under UV illumination (B). Lane M, molecular weight marker (ProSive® color protein markers, BioWhittaker Molecular Applications Inc., USA). Lane 1, sample from ammonium sulfate precipitation; lane 2, sample purified from chelating gel chromatography. The arrow indicates the recombinant chiF protein with the molecular weight at 31.5 KDa. The proteins were trans-blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), and reacted with the chiF antiserum. The existence of chiF was revealed by indirect enzyme-linked immunoassay (C) by use of phosphatase labeled goat anti-rabbit IgG (KPL, Gaithersburg, MD, USA) as secondary antibody. For the plate assay (D), approximately 100 μg of the purified enzyme were applied on the paper disc laid on 0.5% colloidal chitin containing agar. The picture shown was taken 2 days after treatment.

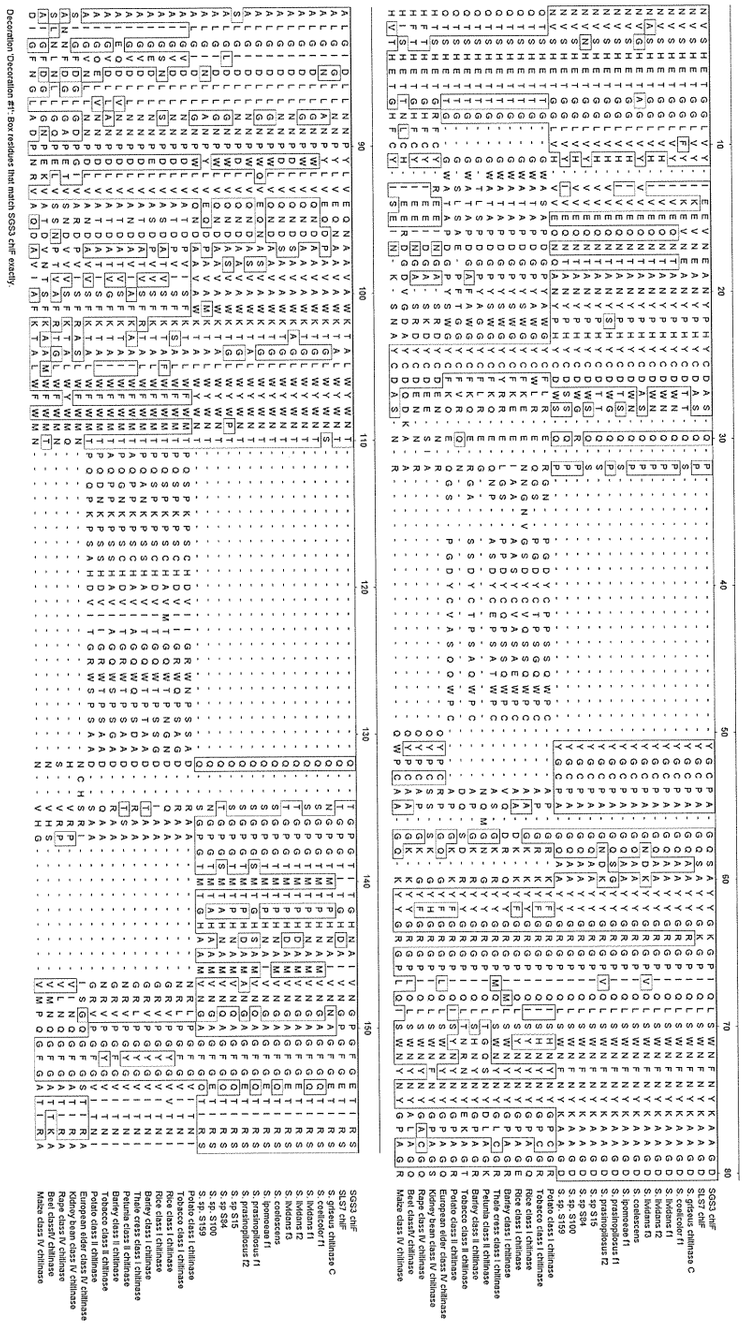

Fig. 9. Pileup alignment of family 19 chitinase genes. Amino acid sequences alignment were processed by MegAlin program of DNASTAR sequence analysis software (DNASTAR, USA) by the method of Clustal W. "-" indicated the amino acid sequence deletion site (gap). The sequences identical to SGS3 chiF were surrounded with boxes.

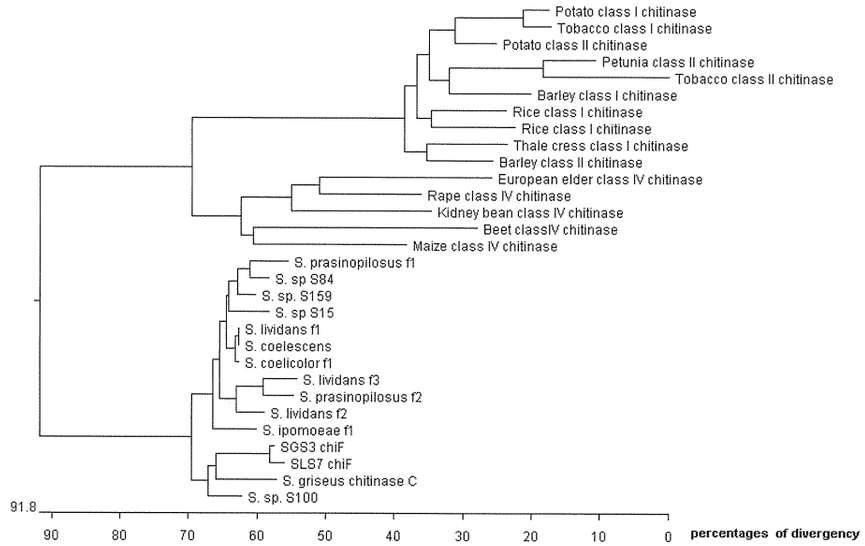

Fig. 10. Phylogenetic tree based on deduced amino acid sequence comparisons of catalytic domain of family 19 chitinase protein.

DISCUSSION

Cloning of chitinase gene aiming for application in plant disease manipulation has been one of the primed research topics during the past decade. The family 19 chitinase, initially discovered exclusively from plant system was known to be a major constituent of pathogenesis related protein (PR protein) with critical importance in induced resistance. Their antifungal characteristics have been well illustrated (Tsujibo *et al.*, 2003). The development of antagonistic *Streptomyces* as bio-fungicide was gaining great attention lately because the difficulty of disease control and the concern on the deleterious impact resulted from chemical pesticide application. The initiative discovery of family 19 chitinase gene *chiC* among members of *Streptomyces* (Saito *et al.*, 2000) and the detection from there-of prepared transgenic rice the enhanced rice blast disease resistance (Itoh *et al.*, 2003) prompted further the exploration regarding to the enzyme application. By PCR amplification with the use of *chiF* specific primer pair, the above shown results demonstrated the presence of the gene among most of the tested antagonistic *Streptomyces* strains (Fig. 3). The existence of *chiF* homologous sequence among these tested strains indicated its popularity among the Taiwan native strains. The failure to detect the homologous sequence from 10 of the tested strains appeared due to the divergence of the gene rather than the absence of the target gene; an adjunct western blot analysis has shown the existence of the enzyme among most of these test strains (data not shown).

For the 891bp full-length sequence of *chiF* cloned, the pair-wise sequence analysis indicated that it shared 87.4, 85, and 73% identity, respectively, comparing to that of *S. griseus chiC*, *N. prasina chiB*, and *S. coelicolor chiF* shown by previous workers. A

common evolutionary origin of the enzyme was clearly indicated. As regards to the promoter sequence which governs the expression of the gene, the direct repeat sequence discovered at the promoter region (Figs. 6 and 7) was similar to that of *S. coelicolor chiF* (Saito *et al.*, 2000); its determinative regulatory role in substrate induction and glucose repression has been depicted (Ni and Westpheling, 1997). In a broth culture of *chiF* transformed *E. coli* Rosetta™ (DE3), the chitin induction and glucose repression of chiF production was demonstrated (data not shown). The promoter with alike direct repeat sequence was found not exclusive only for the family 19 chitinase gene targeted. We recently cloned a full length family 18 chitinase gene *chiA* from the test bacterium SGS3, sequence analysis has shown that it also harbors a direct repeat sequence of TGGTCTGGACCA at the promoter region (Chen *et al.*, unpublished). The *S. griseus* chiC enzyme purified from culture of *chiC* transformed *E. coli* was shown to be greatly inhibitory to the mycelial growth of *Trichoderma resii* (Watanabe *et al.*, 1999). For family 19 chitinase known among members of *Streptomyces*, the unique antifungal characteristics of the enzyme has been attributed mainly to the function of the catalytic domain and also, at least in part, its type 3 chitin binding domain (ChtBD). The type 3 ChtBD was shown consisting of 6 aromatic groups that appeared to be important in substrate binding (Watanabe *et al.* 1999). It was noted that the deletion of ChtBD might impair affinity of the enzyme to the substrate and thus greatly reduced the enzyme attributed growth inhibition (Itoh *et al.*, 2002; Tsujibo *et al.*, 2003). For SGS3 chiF, the chitin binding domain (ChtBD) located at the upstream of *chiF* cloned showed a CBM_5 12 carbohydrate binding domain characteristic and a type 3 ChtBD homologous AKWWTQ sequence identical to that known on *S. griseus chic* and *N. prasina chiB*. The enzyme purified from overexpression of the *chiF*- transformed *E. coli* Rosetta (DE3) broth culture was tested on plate culture against *Trichoderma virens*, *Alternaria* sp., *Rhizoctonia solani* AG4, *Rhizopus* sp. and *Pyricularia oryzae*. Although the sequence data of SGS3 *chiF* (Figs. 2 & 5) was highly identical to *S. griseus chiC* (87.4%) reported (Watanabe *et al.*, 1999), the antagonistic effect was not detected (data not shown). The discrepancy could be due mainly to the use of different microbial strains for gene expression and antagonistic bioassay. In a dual culture assay to illustrate the antifungal activity of SGS3 against various phytopathogenic fungi, remarkable mycoparasitic effect indicating clearly the functioning of chitinases(s) and/or glucanase(s) was consistently detected (data not shown). It seemed likely that the antifungal activity of SGS3 involved synergistic effect of cohorts of lytic enzymes rather than simply a single chiF.

The existence of family 19 chitinase has been shown by Watanabe *et al.*, (1999) a common characteristic among *Streptomyces* species. Since the initial discovery, family 19 chitinase gene was found also present in non-*Streptomyces* members such as *Burkholderia gladioli* (Kong *et al.*, 2001) and *Nocardiopsis prasina* OPC-131 (Tsujibo *et al.*, 2003). Moreover, the phylogenetic analysis of these microbial family 19 chitinase genes have suggested strongly that these genes in *Streptomyces* and *Burkholderia* might be acquired from plant source via horizontal gene transfer (Tsujibo *et al.*, 2003; Watanabe *et al.*, 1999). In the works herein presented, the detection of *chiF* by PCR amplification with the application of *chiF* specific primer +pair (Fig. 3) from most of the tested strains echoed their view in that the existence of family 19 chitinase was indeed a common characteristic among *Streptomyces* strains antagonistic to phytopathogenic fungi. For the 10 test strains which showed negative results in the performed PCR analysis, similar results were obtained in both northern analysis and southern analysis followed (data not shown). However, by western analysis, all these negative strains were detected positive the presence of chiF-antiserum reactive proteins (paper submitted). The immuno-detection results obtained further revealed that these enzyme proteins were serologically related and were mostly nearly identical in molecular weight. The close relevance suggested possibility that they might be evolved from a common phylogenetic origin.

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Population Parameters of *Ganaspidium utilis* Beardsley (Hymenoptera: Eucoilidae) on *Liriomyza trifolii* (Burgess) (Diptera: Agromyzidae)

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ABSTRACT

This study was aimed at determining the population parameters of *Ganaspidium utilis*, a parasitoid of *Liriomyza trifolii*, under the laboratory conditions at 17±2°, 25±2° and 29±2°C. The developmental time of *G. utilis* was longest (32.85±0.86 days) at 17±2°C and shortest (14±0.44 days) at 29±2°C. The developmental time between a male and a female *G. utilis* under the tested temperatures was not significantly different. The longevity of male and female *G. utilis* significantly decreased as the temperature increased. The overall fecundity and sex ratio of the offspring of *G. utilis* were also affected by the variation in temperature. Results of the population parameter analysis showed that 25±2℃ was the most favorable temperature for *G. utilis* to multiply in laboratory conditions. The temperatures at 17±2° and 29±2 ℃ caused lower net reproduction rate of *G. utilis* than 25±2℃. The mean generation time, finite rate of increase and population-doubling time of *G. utilis* had an inverse relationship with temperature; however, the intrinsic rate of increase of *G. utilis* showed a positive relationship with temperature. According to its population parameters, demonstrated in this study, *G. utilis* has a good potential to be an effective natural enemy for the control of *L. trifolii*.

Key words: Parasitoid, *G. utilis*, *L. trifolii*, Population parameters.

INTRODUCTION

Liriomyza sativae (Blanchard), *L. trifolii* (Burgess), *L. huidobrensis* (Blanchard), *L. bryoniae* (Kaltenbach), and *L. strigata* (Meigen) are economically important leafminers (Spencer, 1973). Chien (1997) reported that *L. trifolii*, *L. bryoniae*, and *L. sativae* caused serious damage to ornamentals and vegetables between 1984 and 1995 in Taiwan.

Ganaspidium utilis is a larva-pupal endoparasitoid that was introduced into Hawaii from the Weslaco area of Texas for the control of *L. trifolii* and *L. sativae* (Nakao and Funasaki, 1979). It successfully became established in Hawaii, the Marianas, Tonga, and Guam and has become an important natural enemy of *Liriomyza* spp. (Lai and Funasaki, 1986; Greathead and Greathead, 1992; Johnson, 1993).

In 2003, *G. utilis* was introduced to Taiwan from Hawaii. Upon arrival, the parasitoids were isolated in the quarantine

laboratory of National Pingtung University of Science and Technology (NPUST) and were kept under observation to study their biology and parasitism. Previous studies described the biology of *G. utilis* using *L. sativae* as a host (Petcharat and Johnson, 1988). A cost effective method for the mass production of *G. utilis* was also developed (Rathman *et al.*, 1991). However, life table and population parameters of this parasitoid have not been reported previously. Therefore, the objective of this study was to describe the life table and population parameters of *G. utilis* under laboratory conditions using *L. trifolii* as the host.

Life table studies are fundamental not only to understand demography but also to know the general biology. In such studies, development times and survival rates, longevity of adults and the daily fecundity of females are recorded for every individual (Chi, 1988). Knowledge of the development rate and life history of a parasitoid can be used, along with other developmental parameters such as fecundity and longevity, to estimate the potential impact of the parasitoid on host populations (Martin *et al.*, 1989; Bernal and González, 1993). Life tables can be used to compare the biological traits of parasitoids submitted to variations on biotic and abiotic factors such as temperature, adaptability to host and reproductive potential of parasitoids, and other insect species (Maceda *et al.*, 1994; Pratissoli and Parra, 2000; Pratissoli *et al.*, 2004).

The components of a life table typically include the numbers entering each of several life stages (l_x) in an insects life cycle, numbers dying within each stage (m_x) due to specific factors, together with estimates of rates of lose in each stage (Southwood, 1978). The population parameters as described by Carey (1993) were: the net reproductive rate (Ro), is defined as the mean number of daughters produced per cohort of females over their lifetimes. Similarly, the mean generation time (T) is the average interval separating the births of one generation from the births of the next, the intrinsic rate of increase (r) is the maximum exponential rate of increase by a population growing within defined physical conditions, population increase at each time interval (λ) is defined as the ratio of population sizes at each time step, doubling time (t) is the time needed for a population to double in size from a fixed point in time.

MATERIALS AND METHODS

Using *L. trifolii* larvae as test insects

Throughout this study, the same procedures were followed to obtain the late 2nd or early 3rd instar *L. trifolii* larvae as test insects. Six pairs of *Phaseolus* Henderson lima bean plants (2 leaves each) were placed for 6 hours in a screened cage containing 50~60 *L. trifolii* adults. After 6 hours, the bean plants were removed from the cage and were held for 5 days to allow the *L. trifolii* eggs to hatch and develop until they reached the late 2nd or early 3rd instar.

Rearing of *G. utilis*

Ganaspidium utilis parasitoids used in this study were obtained from a laboratory culture using *L. trifolii* as the host insect. The *L. trifolii* colony was maintained in the laboratory using methods described by Rathman *et al.* (1991). *Ganaspidium utilis* was reared using methods described by Petcharat and Johnson (1988). Stems of *L. trifolii* infested bean plants (2 leaves each) were cut immediately above the roots and placed in a 200 ml flask filled with water. A honey-water solution (25%) was sprayed on the lima bean leaves as a food source for the adult parasitoids. Plants were exposed to *G. utilis* for 24 hours and then removed. The leaves were cut at the base and kept in closed plastic containers to allow the *L. trifolii* larvae to pupate. Leafminer puparia were collected and held in a petri dish (9 cm diameter) until the *G. utilis* adults emerged. Parasitoid adults were returned to the oviposition cages for either culture maintenance or were used in subsequent studies.

The rearing of *L. trifolii* and *G. utilis* was conducted in a laboratory at 25±2°, 50±10 % RH and a photoperiod of 14L: 10D.

Survivorship of *G. utilis*

For this study, 2 lima bean plants infested with late 2nd or 3rd instar larvae of *L. trifolii* were placed into a container, containing 10 pairs of adult parasitoids. After 24 h, exposed leaves were removed and new 50 late 2nd or early 3rd instar leafminer larvae were added. This was done daily until the female parasitoid died. The number of days that a female lived was recorded.

This study was replicated 4 times. Means were compared by using t-test and SNK test of SAS (2003).

Longevity of *G. utilis*

Two leaves containing about 50 late 2nd or early 3rd instar leafminer larvae were used. Leaves were placed in a cage, and newly emerged male and female parasitoids were paired and released into the cage. After 24 h, exposed leaves were removed, and 50 new late 2nd or early 3rd instar leafminer larvae were added. This was done daily until the female parasitoid died. A honey-water solution (25%) was provided to the males until they died. The numbers of days that males and females lived were recorded.

This study was replicated 4 times. Means were compared by using t-test and Student-Newman-Keuls (SNK) test of SAS (2003).

Fecundity and sex ratio of *G. utilis*

Using leaves exposed during the experiment on the longevity of parasitoids, the fecundity and sex ratio were determined. The exposed leaves were held in a petri dish (9 cm diameter) until the leafminer larvae pupated. The puparia were collected and held until adult parasitoids or leafminers emerged. The number of *G. utilis* emerging and the sex ratio were recorded daily. The emerging parasitoids were sexed under a microscope using antennal characteristics as described in Beardsley (1988). The sex ratio was calculated as the fraction of the male offspring to the total number of offspring, as shown in equation 1.

$$\text{Sex ratio} = m/(m+f) \quad (1)$$

Where, m is the number of males produced and f is the number of female offspring produced by *G. utilis* female.

This study was replicated 4 times. Means were compared by using SNK test techniques of SAS (2003).

Determination of population parameters

Data obtained during the study of biological characters of *G. utilis* were used to analyze the population parameters by using procedures reported by Krebs (1978) and Birch (1948). Facing the difficulty in obtaining information on parasitoid development inside the host pupa, the overall percentage of survival of immature stages was estimated by applying the method reported by Rossa *et al.* (2002) by

subtracting the number of *L. trifolii* adults emerged, the number of parasitoids emerged and the number of naturally dead pupae from the total pupae offered.

Net reproductive rate (R_o)

R_o is the average number of female offspring born to the insects considered at age 0. Consider n newborn insects. Some of them will die without producing any offspring (zero offspring), others will produce one or several offspring. R_o is the average number of female offspring produced by a birth cohort of females in their lifetime.

$$Ro = \sum_{x=0}^{n} lxmx \qquad (2)$$

Where, x is age (days); l_x is probability of surviving to age x and m_x is the no. of female offspring born to a mother of age x.

Intrinsic rate of increase (r)

The intrinsic rate of increase (r) is the maximum exponential rate of increase by a population growing within defined physical conditions. The r value was determined by applying the Lotka-Euler equation developed by Birch (1948).

$$\sum e^{-rmx} lxmx = 1 \qquad (3)$$

Where, e = natural base = 2.71878 (a constant).

Mean generation time (T)

The Mean generation time (T) is the average interval separating the births of one generation from the births of the next.

$$T = \log_e(Ro)/r \qquad (4)$$

Where, e = natural base = 2.71878 (a constant) and r = per capita rate of population change

Population doubling time (t)

Population doubling time (t) is the time needed for a population to double in size from a fixed point in time.

$$\ln(2.0) = rt \qquad (5)$$

Where, ln (2.0) is a constant = 0.69315.

Therefore, the time it takes for an exponentially growing population to exactly double in size, regardless of the absolute size of the population will be:

$$t = 0.69315/r \qquad (6)$$

Where, e = natural base = 2.71878 (a constant), r = per capita rate of population change and and t = time in the same units as r.

3.6.5. Finite rate of increase (λ)

Finite rate of increase (λ) is defined as the ratio of population sizes at each time step.

$$Ro = \lambda^T \qquad (7)$$

Where, R_o = Net reproductive rate and T = Mean generation time.

RESULTS

Developmental time of *G. utilis*

The total developmental time of male *G. utilis* significantly decreased as 34.95±1.21 to 13.22±0.2 days when the temperature increased from 17±2°C to 29±2°C. This trend was also observed in case of male *G. utilis* too. The total developmental time of male *G. utilis* significantly decreased as 30.75±0.95 to 15.37±0.75 days when the temperature increased from 17±2°C to 29±2°C. The days required for males and females of *G. utilis* offspring to emerge from puparia were not significantly different at the temperatures tested (Table 1).

Longevity and fecundity of *G. utilis*

The males and females of *G. utilis* lived longest at 17±2°C and shortest at 29±2°C. There was no significant deference in the longevity of male and female *G. utilis* across the temperatures (Table 1).

Results of this study on the fecundity of a *G. utilis* female revealed that the highest number of male and female offspring produced was at 29±2℃. The total number of progeny produced was highest and lowest at 29±2° and 17±2℃, respectively. The total number of progeny produced at 29±2° and 25±2℃ was not significantly different. The sex ratio of newly emerged progeny decreased from 0.62 to 0.59 as temperature increased from 17±2° to 29±2℃ (Table 1).

Population parameters of *G. utilis*

The *G. utilis* females demonstrated similar survival trends across the temperatures (Fig. 1). Mortality of *G. utilis* females occurred faster at 29±2°C than at 25±2° and 17±2℃. The short adult lifespan at 29±2℃ suggested that *G. utilis* was more sensitive to warmer temperatures. The extended longevity of adult *G. utilis* at 17±2℃could be explained by reduced activity, less energy utilized for reproduction, or both. *G. utilis* females did not produce progeny at first 2 days after emergence at 17±2°C; however, *G. utilis* females produced progeny immediately after the emergence at 25±2° and 29±2℃ (Fig. 1). Daily progeny production was not uniform across the temperatures. Progeny production peaked during the first few days of adult life and then declined sharply at 17±2° and 25±2℃ and declined gradually at 29±2℃. The highest progeny production was attained on day 5 (2.5 progeny/female/day) at 17±2℃, day 6 (6.8 progeny/female/day) at 25±2℃ and day 2 (7.75 progeny/female/day) at 29±2℃ (Fig.1). With in the half-life of *G. utilis* female, about 71, 64 and 74 % of the progenies were produced at 17±2°, 25±2° and 29±2°C, respectively.

The life table of *G. utilis* demonstrated an inverse relationship between the mean generation time (T) and the temperature (Table 1). T was highest at 37.33 days at 17±2°C decreased to 22.93 days at 25±2°C and then to 18.28 days at 29±2°C (Table 1). The net reproductive rate (R_o) of *G. utilis* was highest at 33.55 at 25±2°C, then followed by 26.22 at 29±2°C and 6.17 at 17±2°C. The finite rate of increase (λ) of *G. utilis* showed a positive relationship with the temperatures. *G. utilis* demonstrated highest finite rate of increase (number of females added to the population per female) at 29±2°C. The intrinsic rate of increase (r) of *G. utilis* increased with the increase of temperature. At 17±2°C, r was 0.049, which increased 3.2 times (0.157) at 25±2°C and 3.67 times (0.18) at 29±2°C, and the maximum capacity of population increase was at 29±2°C. Population doubling time was shortest at 3.85 days at 29±2°C, which increased to 4.41 days at 25±2°C and 14.46 days at 17±2° C (Table 1).

DISCUSSION

Chien and Ku (2001b, c) reported that the longevities of *H. varicornis* were 22.4 ± 0.6 (female) and 14.5 ± 1 days (male), and for *N. formosa* were 22.3 ± 1.2 (female) and 7.5 days (male) at 25°C. Similarly, Lopez *et al.* (2004) reported that the longevities of *Halticoptera circulus* (Walker), an imported parasitoid form Hawaii to Taiwan, were 34.25 ± 4.92 (female) and 14.25 ± 2.36 days (male) at 26°C. Petcharat and Johnson (1988) also observed longevities of 8.9 ± 1.4 (male) and 8.8 ± 2 days (female) for *G. utilis* at 26°C. The longevity of females observed in this study was higher than that reported by Petcharat and Johnson (1988) but lower than the rest of the other parasitoids mentioned above at both temperatures.

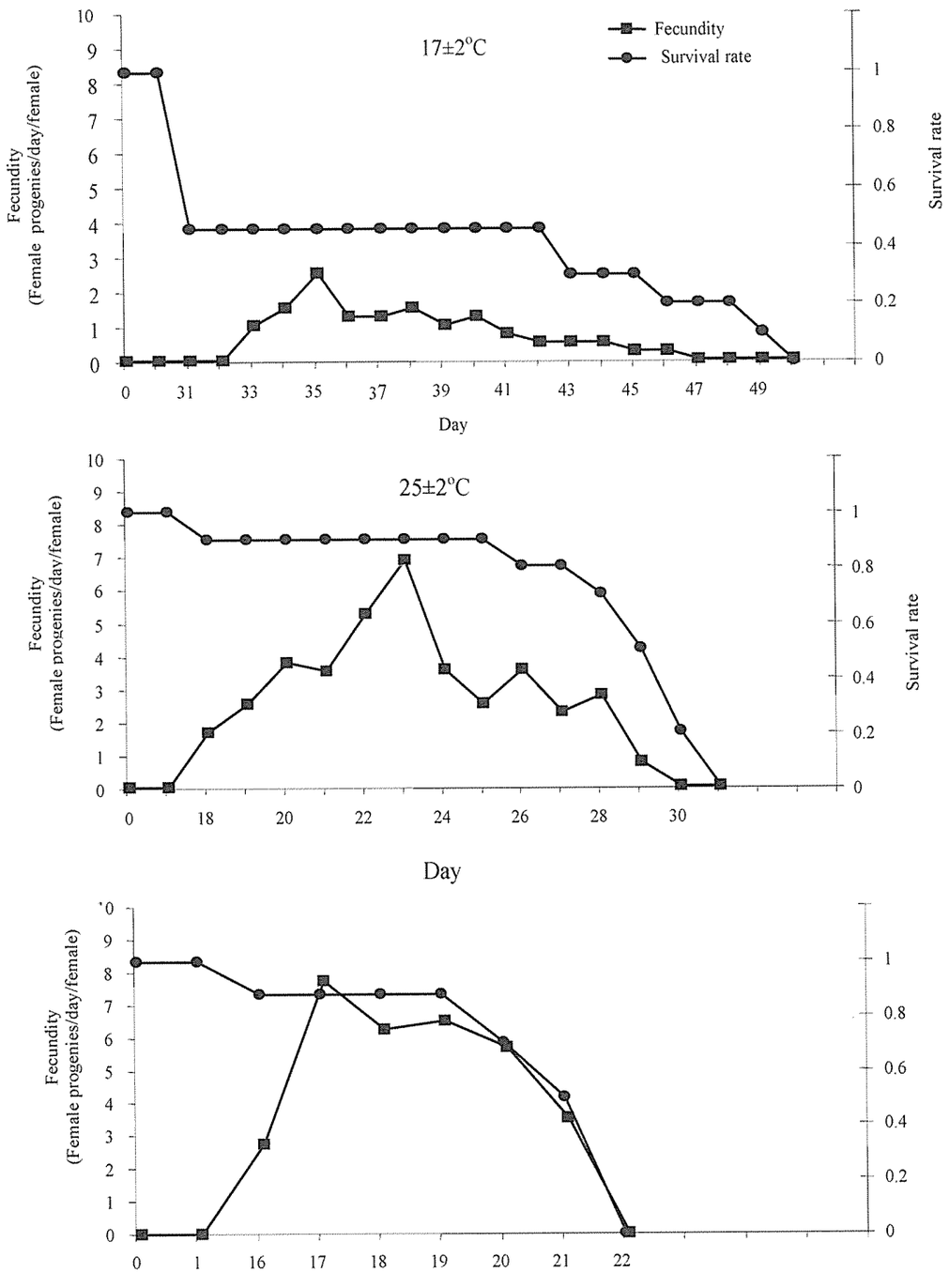

Fig. 1. Fecundity and survivorship of *G. utilis* at different temperatures.

Table 1. Life history parameters of *G. utilis* at different temperature.

Parameters	17±2°C*	25±2°C	29±2°C
Developmental time (days)			
Male	34.95±1.21Aa	18.83±1.59Ab	13.22±0.2Ac
Female	30.75±0.95Aa	17.33±1.26Ab	15.37±0.75Ac
Longevity (days)			
Male	16.5±2.08Aa	10.5±3.42Ab	5.25±1.26Ac
Female	19±2.16Aa	12.87±2.9Ab	7.12±1.93Ac
Progeny produced			
Male	23±6.27Ab	27.79±8.35Bb	46±4.32Aa
Female	14±3.1Ab	38.87±8.66Aa	32.5±3.1Aa
Total	37±9.05b	66.67±8.5a	78.5±7.1a
Sex ratio (male/male + female)	0.62	0.42	0.59
Population parameters			
Net reproductive rate R_o	6.17	33.55	26.22
Mean generation time (days) T	37.33	22.93	18.28
Finite rate of increase λ	1.05	1.17	1.19
Intrinsic rate of increase r	0.049	0.157	0.18
Population doubling time (days) t	14.46	4.41	3.85

* Means followed by different letters in the same row (lower case) or in the same column (upper case) are significantly different by SNK test ($P<0.05$).

Developmental times varied between *H. varicornis* (11.1 ± 0.1 days) and *N. formosa* (14.4 ± 0.2 days) at 25°C (Chien and Ku 2001b, c), and *H. circulus* (31.57 ± 4.49 days) and *G. utilis* (25.9 ± 1.4 days) at 26°C (Petcharat and Johnson, 1988; Lopez *et al.*, 2004). The developmental time observed in this study was lower than that of *G. utilis* observed by Petcharat and Johnson (1988) and *H. circulus* by Lopez *et al.* (2004), but was similar to or higher than those of *H. varicornis* and *N. formosa* (Chien and Ku, 2001b, c).

The mean number of progeny produced per female also varied with the parasitoid species at 204 ± 22 (*H. varicornis*) and 202 ± 18 (*N. formosa*) at 25℃ (Chien and Ku, 2001b, c), and 145.25 ± 0.67 (*H. circulus*) and 51.4 ± 13.8 (*G. utilis)* at 26℃ (Petcharat and Johnson, 1988; Lopez *et al.*, 2004). In comparison with other parasitoids, the progeny production observed in this study was higher than that reported by Petcharat and Johnson (1988) and lower than the other reports at both temperatures.

The female sex ratios were reported to be 0.62 *(H. varicornis*) and 0.54 (*N. formosa)* at 25°C (Chien and Ku, 2001b, c), and 0.67 (*H. circulus)* and 0.58 (*G. utilis*) at 26°C (Petcharat and Johnson, 1988; Lopez *et al.*, 2004). The female sex ratio of *G. utilis* observed in this study (0.58) was similar to that observed by Petcharat and Johnson (1988), but higher than that of *N. formosa* at 25 ± 2°C (Chien and Ku, 2001c).

Because of the highest net reproductive rate, detected at 25±2°C, this temperature was the most favorable temperature for multiplying the *G. utilis* in laboratory (Table 1). Because of the shortest longevity, shortest developmental time and highest intrinsic rate of increase, the population of *G. utilis* could be doubled in 3.85 days at 29±2°C (Table 1). Many researchers reported that the optimal temperature of a parasitoid can be expressed by their optimum growth and development, fecundity and longevity at

certain temperature ranges. It is also reported that the temperature affects negatively on average generation time, finite rate of increase and population doubling time and positively on the intrinsic rate of increase of parasitoids (Froud and Stevens, 1997; Olaye *et al.*, 1997; Pratissoli and Parra, 2000; Olaye *et al.*, 2001; Pratissoli *et al.*, 2004).

At 25℃, the intrinsic rate of increase (r) for *L. trifolii* (0.2329) was, higher that the r value of *G. utilis* (0.157) (Chien and Ku, 1996). This resulted the several overlapping generations in the same population of *L. trifolii*. Therefore, *G. utilis* females can easily find host to lay their eggs. Based on the results, the intrinsic rate of increase of *G. utilis* that obtained from 3 different temperatures were lower as compared with other hymenopteran parasitoids including *Tamarixia radiata* (0.2948) (Chein *et al.*, 1994), *Tekenomus isis* (0.203) (Olay *et al.*, 2001), *Trichograma pretiosum* (0.32), *T. acacioi* (0.35) (Pratissoli and Parra, 2000), *T. busseolae* (0.258) (Olaye *et al.*,1997) and *Thripobius semiluteus* (0.1101) (Froud and Stevens, 1997). Based on the results, the intrinsic rate of increase of *G. utilis* that obtained from 3 different temperatures were positively co-related with temperature. The positive effect of temperature on the intrinsic rate of increase were also reported by many researchers for other parasitoids including *T. radiata* (Chein *et al.*, 1994), *T. isis* (Olay *et al.*, 2001), *T. pretiosum*, *T. acacioi* (Pratissoli and Parra, 2000), *T. busseolae* (Olaye *et al.*, 1997) and *T. semiluteus* (Froud and Stevens, 1997).

CONCLUSIONS

As it was demonstrated in this study, depending upon the temperature, a female of *G. utilis* lives between 1 to 3 weeks and lays eggs throughout its lifespan. Once *G. utilis* population established in field, the colonization and dispersal will be fasters because *G. utilis* population could be doubled within 4 to 14.5 days, depending upon the temperature. Because of the shorter population doubling time, several generations of *G. utilis* could be overlapped in a population. This attribute may be beneficial to the control of *L. trifolii* as its population also known to be overlapped in the field. Because of the lower female sex ratio, it would require more *G. utilis* females being released in the field to achieve a significant control of *Liriomyza* leafminers. Based on the biological characteristics and parasitization performance observed in this study *G. utilis* can be considered as a potentially effective biological control agent of *L trifolii* at 25±2 and 29±2°C, which are favorable for the activities of *G. utilis*.

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Area-wide Control of the Oriental Fruit Fly and Melon Fly in Taiwan

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ABSTRACT

Area-wide control have been tested for the oriental fruit fly (*Bactrocera dorsalis* Hendel, OFF) and melon fly (*Bactrocera cucurbitae* Coquillett) in Taiwan. The control tactics depend mainly on methyl eugenol, cuelure, and protein bait containing spinosad. The non-chemical efforts are concentrated on (1) organize involved farmers, (2) establish geological information, (3) monitor fruit fly density, (4) establish fruit production information (5) hold training programs and (6) maintaining the orchard sanitation. Area-wide OFF control had been tested from the year of 2000 to 2004 in 21 districts, and the result indicated that farmers with common interest, mainly in a monocrop system, will cooperate better than those planting multiple crops. Taitung area presents a fruitful result due to sugar apple, *Annona squamosa* L., constitutes 95% of its fruit production, and OFF density ratio regulated in inner quarter, buffer zone, and outskirt is 1 : 10 : 30-100. Within three year, control acreage in Taitung has expanded from 4000 ha to 10000 ha and then to 15000ha. Although GIS/GPS technologies have been used in density monitoring and control strategy planning, 3-dimentional aerial photo have gained stronger echo from the local farmers. The 3-D digital displayed system creates a "virtual reality" of targeted areas on screen which ignites farmers' interest because they can overlook their own orchard and the whole control area. Bring the whole area into a conference room has contributed to the enthusiastic participation in the ongoing area-wide control program, combining the pest density data also give farmers a plan of work, and more details on control strategy can be discussed.

Key words: Area-wide control, Bactrocera dorsalis, Bactrocera cucurbitae.

INTRODUCTION

The fruit fly control project was improved by (1) establishing an island-wide 10-day interval's fruit fly density monitoring system since 1994, (2) controlling the quality of the lure, and (3) testing better field application strategies. By suggesting correct suppression measures for farmers, the oriental fruit fly population has been suppressed to a level of 15 % of pre-control density. These efforts were supported by the funding from government for purchasing 40 tons methyl eugenol (ME) per year, and then manufactured more than two millions ME fiberboard baits to cover 150,000 ha orchards. Fruit crops protected are mango, wax apple,

citrus, guava, peach, pear, sugar apple, carambola, etc.

In this project, we continuously exchange ideas with the Hawaiian fruit fly control working team. The new approaches applied in the area-wide control strategies include the improvement of ME baits, incorporate new traps and protein bait, GF-120. We successfully organize 155 farmer associations to synchronize the fruit fly control project. However, numerous technology developed in recent years have been incorporated into the program, particularly, the GEO information as well as computerized data processing in pest monitoring and damage assessment.

MATERIALS AND METHODS

1. Attractants and baits used: methyl eugenol, cuelure, GF-120 and other protein hydrolysate baits.
2. Test insects: oriental fruit fly and melon fly.
3. Traps: improved trap using fiber board/cloth was designed, which can periodically reload fresh ME bait mixture.
4. Monitoring trap: the pheromone trap for *Spodoptera exigua* has been used.
5. Poisoned baits: 95% naled mixed with insect attractants (methyl eugenol or cuelure) at the ratio of 5 to 95.
6. Forecasting model construction and analysis of population change rate.
7. New software for GIS/GPS systems.
8. Aerial photos combining with the 3-dimentioanl digital display software.

RESULTS AND DISCUSSION

Establish Area-wide Fruit Fly Control Districts

With the cooperation of the Hawaiian fruit fly working team, the official fruit fly control program sponsored by the Bureau of Animal and Plant Health Inspection and Quarantine (BAPHIQ), this area-wide (AW) control program has advanced rapidly, particularly on the establishment and extension of AW control strategy. For example, starting in 2002 after intensively field survey and discussion with the farmers and staffs of Taitung Farmer Association, the OFF density monitoring has been initiated for their AW control district.

Taitung is the earliest established AW control district, which initially covering an area of 4,000 ha, and was expanded to 15,000ha in 2005. The frontier, the buffer zone and the inner circle were established, and each has its own density data to realize the fruit fly invasion from the western mountainous side. Fruit fly of frontier, buffer zone an inner circle has a density gradient of 30-100 : 10 : 1, and the fruit fly damage in Taitung has been minimized in sugar apple (Fig. 1).

The active "search and destroyed" monitoring tactic has also been tested since 2002, in another 15 control districts covered a total of more than 10000 ha in the west coast (Fig. 2). Amongst these areas, Tou-lieu AW district is the most successful district with outer and inner OFF density difference exceeds 70 times. However, other AW districts still not able to reach the first stage implementation target, i.e., a differences of 3 to 10 times.

In 2005, four new districts are established, which located in Hua-lien in east coast of Taiwan, and one hundred km north from Taitung. Only in their first year of OFF control, the farmers have already gained success result in pomelo.

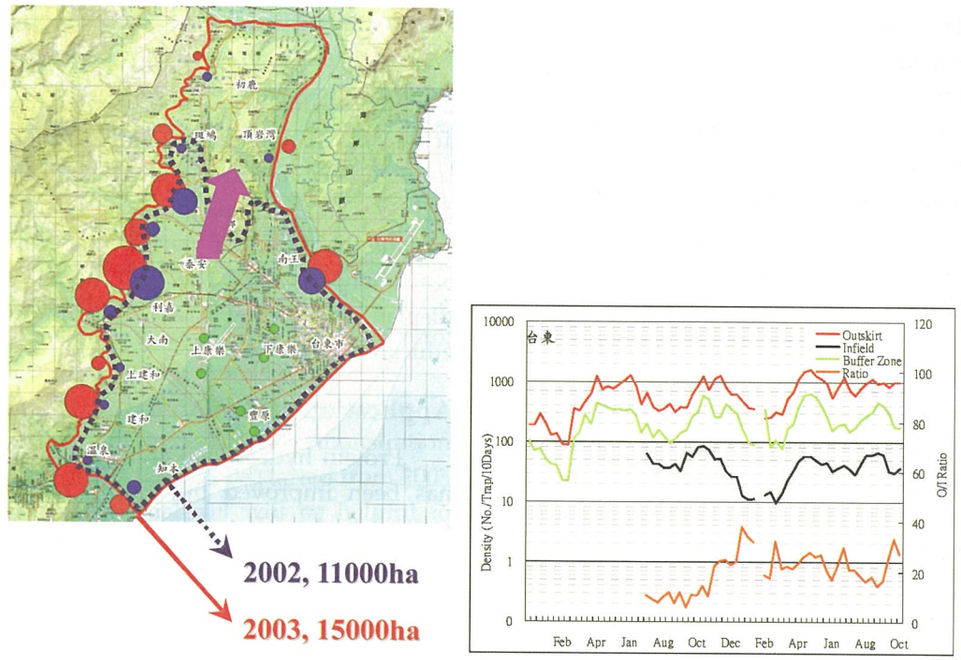

Fig. 1. Oriental fruit fly density monitored in Taitung AW control district in Dec. 2003 (innermost: green spot; inner: blue spot; outer: red spot).

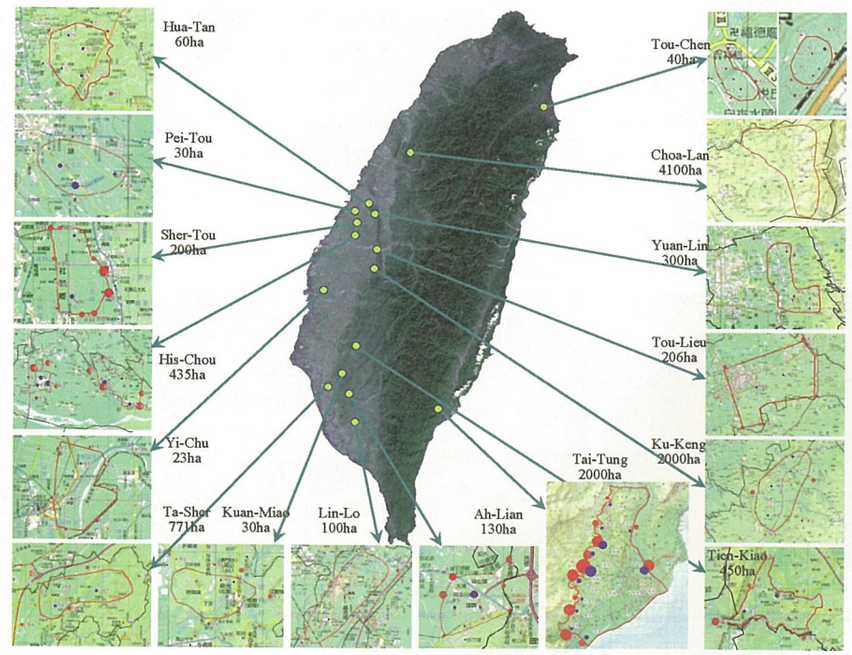

Fig. 2. Sixteen established OFF AW control districts in Taiwan, 2002-2004.

Tree other new control districts are in Chia-yi County, and the acreage of each district does not exceed 1,000 ha., but the OFF control results have exceeded 15 previously established districts in west coast. The main reason for this success is due to the incorporation of 3-D aerial photo digital system in the meeting of planning and designing the control strategy with the farmers. This new advance has been considered as a breakthrough to our previous believes that the AW control area should be as large as possible, and can not be succeeded in a small area. Through detailed discussion and planning, and with the help of 3-D aerial photo illustration, farmers of these small control districts are more enthusiastic, and enable us to get more involvement of farmers in OFF control details (Fig. 3).

Farmer Education

Farmer education program has been continued. The cage for demonstrating the actions of ME and cuelure on FF and MF has resulted very convincing visual confirmation of the bait's action to farmers, and has become an essential part in the farmer's education session. The 3-D aerial photo display also offers great help in farmer education sessions.

Application of Protein Baits

Protein hydrolysate application method has been improved by adding borax and sodium hydroxide to increase the pH value, which will increase the ammonia component of food bait and resulted in better trapping effect. Procedures for blending the mixture is well illustrated in a pamphlet and dispatched to 155 farmer associations.

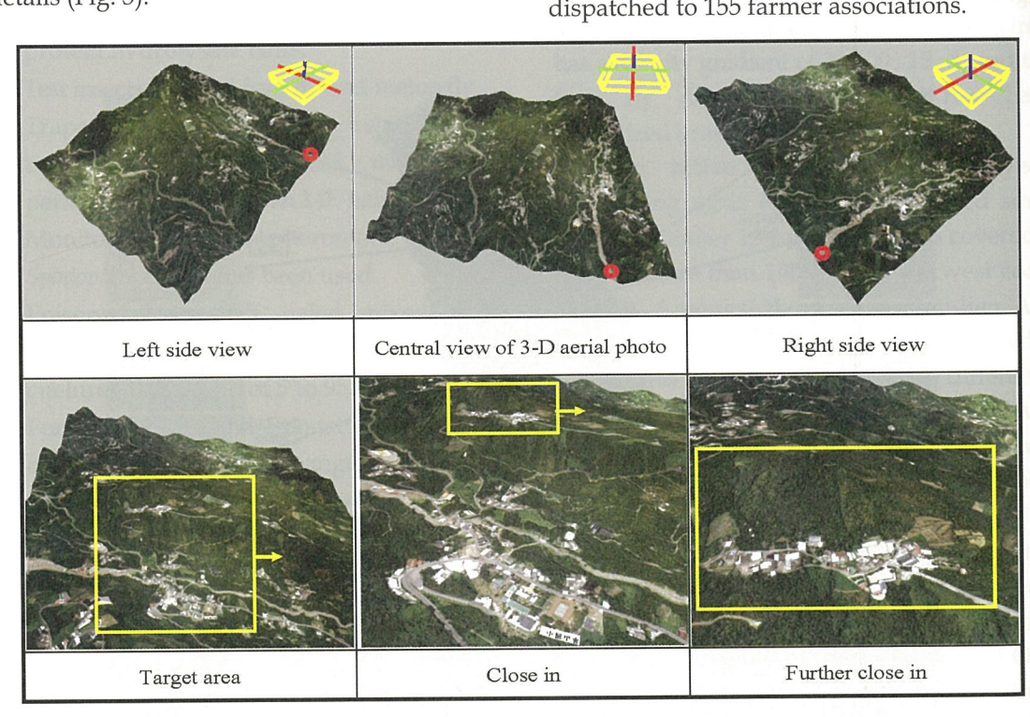

Fig. 3. The newly established AW control district, Chuen-nan Village, shown in the 3-D digital display system.

With the help of Dr. Ronald Mau and the Dow Chemicals, the new protein bait, GF-120, has been successfully introduced to Taiwan in 2003. Two demonstration orchards have applied GF-120 and compared to the traditional protein baits for education and extension purposes. The effects of GF-120 are also introduced to several districts that produce luffa, melons and cantaloupe for melon fly control, and current peak control period is spring to summer (Fig. 4).

GF-120 has received its registration in Taiwan in 2005. Official demonstration program for GF-120 will be started from 2005 and this useful material will be helpful to reduce the female fruit fly density in an OFF outbreak and also reduce the environmental hazard of chemical insecticides.

Orchard Sanitation

Enhance field sanitation has been conducted in the four newly established AW control districts in both Hua-lien and Chia-yi Counties in 2005 (Fig. 5).

Issuing the OFF Newsletter Every Ten days

The island-wide OFF monitoring network continuously provides population census data, which enables the working team to analyze OFF population dynamics and to construct the forecasting model. These analytical works proved to be useful in identifying high-density location and season when OFF increase rate reach the high level. The obtained information then feedbacks to the local farmer associations in a pre-warning system for fruit fly control on the newsletter in 10-day interval (Fig. 6). In our planning, all those information will be available at the ARI's website from September, 2005.

Fig. 4 Field discussion on the application of GF-120 on trap crops.

Fig. 5. Plastic containers provide to farmer for field sanitation.

Fig. 6. Newsletter published every 10 days showing fruit fly density in different areas and the density differences in established AW control districts.

The density of the oriental fruit fly for the past 12 year has under the ME bait control distributed by the government. Although AW control districts have only been established for 3-4 years, the know-how of new technology developed in this program has been refined through the corporation of Hawaiian researchers. At the end of four years's program, the sign of success of this modernization effort is demonstrated clearly in both large district such as Taitung, as well as the small districts in Chia-yi County.

Complete AW control research work will be documented in a standard operation manual by ARI with the opinion and comments from Hawaiian research Group. The manual will serve as a frame work and guideline for future OFF control work in different situations and may also serve as the preparation step for fruit exportation through quarantine.

The farmer's group will always be invited in the meetings to discuss their success and failure, particularly in the conclusion meeting of this COA/USDA collaboration program held in October 12-13, 2005 in Taiwan. Invited participants also included Dr. Ronald F. L. Mau, Dr. Eric B. Jang and Dr. Roger I. Vargas.

The Taiwan experience in this program has gained the recognition of Hawaiian fruit fly working group and had been invited to the 5th APCE meeting held Jeju, Korea as the co-chair in the fruit fly session, and to exchange the experiences obtained in this cooperative research project conducted during 2000-2004 with participants from other countries.

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Spider Mite Damage Mapping and Cotton Canopy Biophysical Characterization Using Spectral Mixture Analysis

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ABSTRACT

Spectral mixture analysis (SMA) is an analytical tool that can identify and measure the components that make up mixed pixels. Used with hyperspectral remotely-sensed (HRS) imagery, SMA permits mapping of surface components from research plot to landscape scales. These technologies are being used increasingly in agronomic research and have potential for precision agriculture to map spatially and temporally variable crop conditions and stresses. SMA was applied to two data sets collected from different hyperspectral imagers. For both studies, radiometrically-calibrated crop and soil spectra were collected using ground-based spectroradiometers and imagers to provide reference spectra. In one experiment, strawberry spider mite (*Tetranychus turkestani* U.N.) damage in cotton was mapped. This pest causes leaf puckering and reddish discoloration and can reduce boll yield. Analysis showed that pest damage could be successfully identified, the relative abundance mapped in large research plots, and temporal changes in damage were consistent with known ground conditions. One confounding factor was shadows which interfered with proper abundance estimation of mite damage. To understand the effects of shadows on estimating canopy characteristics, a second study was conducted where spectra of sunlit and shadowed leaves and sunlit and shadowed soil were measured in cotton research plots. The intent was to determine whether SMA and HRS could simultaneously measure percent cotton cover, height, and chlorophyll concentration and whether the influence of shadows could be isolated. Results showed that including shadows as reference spectra improved relationships between imagery and ground measurements. Mapping sub-pixel landscape components based on spectral signatures could be useful for crop and ecosystem models, irrigation scheduling, locating water and nitrogen stress, and simultaneous measurement of key plant and crop parameters.

Key words: Spectral mixture analysis, Hyperspectral remote sensing, Precision agriculture, Cotton, Spider mites.

INTRODUCTION

Spectral mixture analysis (SMA) is one

type of advanced image processing technique that has been used to analyze hyperspectral imagery from ecological studies for a number of years, and the procedure is well documented (Adams and Smith, 1986; Roberts, *et al.*, 1998; Okin, *et al.*, 2001). A few manuscripts have addressed the issue of detection or discrimination of field- or canopy-level features in agricultural crops with hyperspectral remote sensing (Palacios-Orueta and Ustin, 1996; Gat *et al.*, 2000; Estep and Davis, 2001; Fitzgerald, *et al.*, 2004a). However, using spectral unmixing for estimating crop biophysical characteristics or arthropod detection is not well established.

Linear spectral unmixing is based on the assumption that each pixel is a physical mixture of distinct components (endmembers), and the spectrum of this mixed pixel is a linear combination of the endmember reflectance spectra (Tompkins, *et al.*, 1997). SMA assumes that a small number of endmember spectra can describe most of the spectral variation in a scene and be used to "unmix" the pixels to determine the fractional abundance of each component within a pixel. A modified SMA, the Multiple Endmember Spectral Mixture Analysis (MESMA) allows each pixel to be modeled by a variable number of endmembers (Roberts, *et al.*, 1998). Either SMA or MESMA could allow discrimination of one plant stress from another through identification of differences in spectral curves and then quantify the amount present in each pixel. Relationships can also be developed between the imagery and canopy parameters, allowing simultaneous estimation of canopy cover, height, chlorophyll concentration, or other traits. Fractional abundance maps produced could indicate both the spatial extent and severity of particular stresses or amount of a particular canopy parameter. Such maps would permit a farm manager to locate a stress precisely in a field, thereby providing guidance for field scouting and precision application of pesticides or fertilizer. They could also be used as inputs to crop, irrigation, carbon cycling or other models to permit spatial modeling.

This report describes the real-world application of hyperspectral remote sensing and SMA from two USDA-ARS published studies. In one, the severity and extent of damage to a cotton crop caused by the strawberry spider mite (*Tetranychus turkestani U.N.*) is demonstrated. In a second study, cotton cover, height, and chlorophyll *a* (Chl *a*) concentration were estimated.

MATERIALS AND METHODS

Methods are fully described in Fitzgerald *et al.* (2004a and 2005). A brief description is provided here.

Spider Mite Study

An experiment was established on two, 2.8 ha fields at the USDA-ARS research station in Shafter, CA (35.5° N, 119.3° W., 120 meters above sea level). Each field was planted to cotton (*Gossypium hirsutum* L. variety "Maxxa") on 4 May 1999. Both were managed according to standard cultural practices for cotton in the area except in one (Field 41) spider mites were allowed to prosper by applying a broad acting pesticide, reducing beneficial predators. The other (Field 42) was treated with pesticides to control spider mite infestations following standard local practices.

Spectra of ground components were collected using a liquid-crystal tunable-filter imaging system (Fitzgerald *et al.*, 2004a; Fitzgerald, 2004b). Components included sunlit healthy leaves (H), sunlit mite-damaged leaves (M), tilled sunlit soil (S), tilled shaded soil (Sh), opened cotton boll (CB), and dead leaf (D) (Fig. 1). These were selected based on observation of the principal canopy components. Reference spectra of these components were constructed by selecting representative pixels on leaves and soil. Each image was acquired within one hour of solar noon. Images were corrected to reflectance using a 99% Spectralon calibration panel (Labsphere, North Sutton, NH, USA), which was placed in the field of view before and after image acquisition.

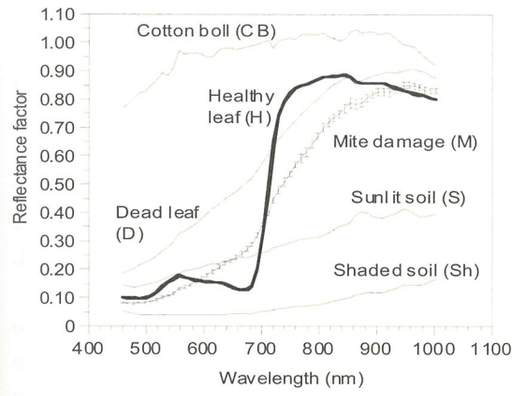

Fig. 1. Six reference spectral signatures for the spider mite study. The error bars on the mite spectrum represent ±1 standard error.

Imagery of the research fields was acquired under clear skies from NASA's Airborne Visible Infrared Imaging Spectrometer (AVIRIS) on four separate dates in 1999. The hyperspectral imagery was composed of 224 images acquired contiguously from 400-2500 nm in 10 nm bands. Those bands matching ground spectra were used in the analysis in the range 459 to 1002 nm. The AVIRIS data sets were atmospherically corrected and converted to reflectance. Ground pixel resolution was 18 m.

Cotton Canopy Components Study

Cotton (*Gossypium hirsutum* L., variety Deltapine 458BR) was planted on raised beds in a 2.5 ha field at the Maricopa Agricultural Center (33.073 N; 111.979 W, 350 m above sea level) in Central Arizona on 7-8 Apr 2003 (DOY 97-98). The beds were spaced 1.02 m (40 in) apart, in a north-south orientation. The cotton germinated beginning on 16 Apr 2003 (DOY 106), was defoliated on 18 Sep (DOY 261), and harvested in mid-October.

The field was divided into 32 plots (each 11.2 by 20 m), which were assigned to 12 different experimental treatments including three plant density and two N level (high and low), which provided a range of variation comparable to a grower's field. The plant density sub-treatments consisted of: Sparse (5 plants/m2, single-line planting); Typical (10 plants/m2 in single-line planting); and Dense (20 plants/m2, double-line planting). The two fertilization levels were: High N (split N applications, based on optimum local practices); and Low N (no additional N applied as fertilizer). A set of smaller plots with unplanted wet and dry soil as well as cotton grown under two different levels of chronic water stress were provided as extremes for measurement of soil and canopy variables.

Hyperspectral imagery was acquired under clear sky conditions from either a light aircraft or helicopter on five dates in 2003. The imagery was acquired with the Portable Hyperspectral Tunable Imaging System (PHyTIS), which captures images in discrete wavebands from 400 to 1100 nm (Fitzgerald, 2004b). Ground pixel resolutions ranged

from 0.55 to 1.2 m. Images were corrected for vignetting, co-registered (Erives and Fitzgerald, 2005), and converted to reflectance using four, 8 m X 8 m reference tarps visible in the scene. The wavelength range of the final imagery used for analysis was from 440 nm to 810 nm with 10-nm sampling interval and spectral resolution.

The canopy components, sunlit leaves (LS), shadowed leaves (LSh), sunlit soil (SS), and shadowed soil (SSh) were measured in the field with an ASD Full Range spectrometer (Analytical Spectral Devices, Boulder, CO). The sensor was mounted on a 1.5 m pole held nadir to the component of interest to emulate the view angle of the PHyTIS airborne sensor platform. The radiance data were converted to reflectance using a 99% Spectralon panel.

Cover was measured photographically with a digital camera. Height was measured non-destructively in the field using a meter stick. Chl *a* concentration was determined using the 80% acetone-BHT method according to Lichtenthaler and Wellburn (1983) on the third fully-expanded leaf from 12 plants per plot, one leaf per plant.

Mixture Analysis

For both studies, SMA was performed using the built-in Linear Spectral Unmixing (LSU) tool in the ENVI software (Research Systems, Inc. Boulder CO). Since this does not account for variable endmember numbers per pixel, the abundance fractions from each region were then exported for further analysis using a spreadsheet-based MESMA model.

The simple linear mixture model takes the form (Roberts *et al.*, 1998):

$$P'_{i\lambda} = \sum_{k=1}^{N} f_{ki} * P_{k\lambda} + \varepsilon_{i\lambda} \text{ and } \sum_{k=1}^{N} f_{ki} = 1, \quad (1)$$

Where $P'_{i\lambda}$ is the spectral mixture at location i at a given wavelength λ modeled as the sum of N reference endmembers, $P_{k\lambda}$, with each weighted by the fraction, f_{ki}. The unmodeled portion is the residual term, $\varepsilon_{i\lambda}$ and the endmember fractions are constrained to sum to 1.

Selection criteria of valid two, three or four endmember sets for each region are described in detail in Fitzgerald *et al.* (2005).

RESULTS AND DISCUSSION

Spider Mite Study

There were statistically significant differences between the two fields for mite counts (Field 41 = 4.9 and Field 42 = 3.1 mites/10 leaves) as determined by a t-test at α = 0.05 (p=0.0002). Spectral unmixing showed that Field 41 had intense mite damage while Field 42 was free of mite damage during the season. This matched ground observations. When the mite, plant, and soil fractional abundance images are composited these image components can be mapped (Fig. 2). Mean mite abundance fractions from the 13 Jun image showed no differences between the fields early in the season before mites had entered the fields (gray). The 28 Aug and 1 Sep images show that Field 41 had significant mite damage (dark gray) but Field 42 was mite free, showing only healthy vegetation (light gray). The final 24 Sep image shows that once senescence began in the cotton, the reddish leaves mimicked the signature of mite damage and thus could also be mapped (dark gray). Late season re-growth of cotton in field 41 is apparent by the light gray color at the west edge.

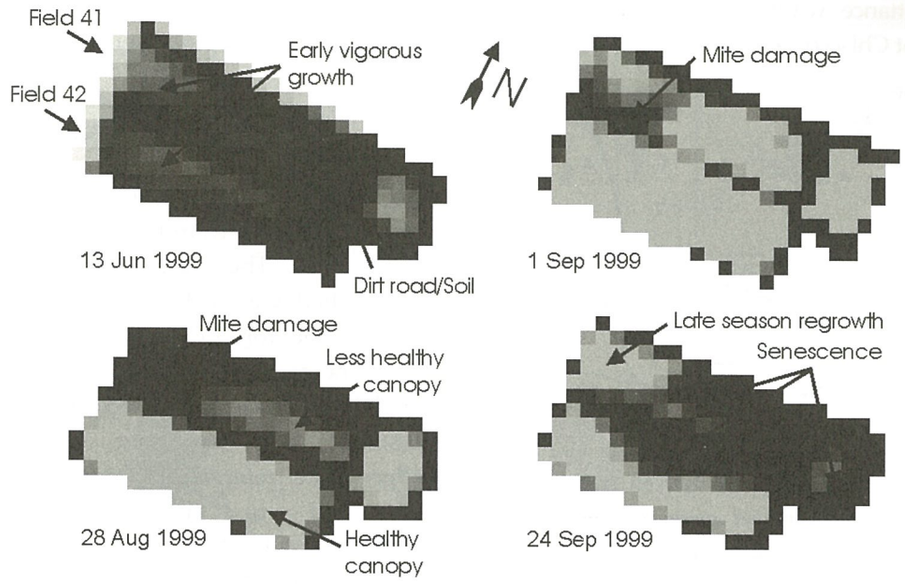

Fig. 2. Composite images normalized for shade of Mite (dark gray), Healthy (light gray), and Soil/Road (black, edges) fractions showing changes across the season. The dark gray areas could direct a farmer to mite damaged areas. The smaller field to the east was managed the same as Field 42.

Shadows were a confounding factor in estimating fractional abundances. Mite fractional values were 0.09 and 0.01, respectively for Field 41 on 28 Aug and 1 Sep. Since these images were acquired only four days apart they would be expected to have similar fractions. Mite fractions were normalized for shadows by dividing by (1-shadow fraction), which tends to remove the influence of shading (Adam *et al.*, 1993). Normalized for shadows, the mite fractions changed to 0.20 and 0.04, respectively, a 5:1 ratio. This only partly corrected for shadows probably because the only shadow fraction incorporated into the analysis was soil shadowing. This was investigated in the following study.

Cotton Canopy Components Study

Since it was shown in the spider mite study that shadows had a confounding effect on the measurement of important canopy components, it was hypothesized that including a self-shadowing reference spectrum would reduce this confusion. The principal spectra in any vegetated canopy are represented by the spectra presented here, LS (Leaves, Sunlit), LSh (Leaves, Shadowed), SS (Soil, Sunlit), and SSh (Soil, Shadowed) [Fig. 3]. The estimations of biophysical and chemical characteristics presented here, canopy cover, canopy height, and Chl *a* concentration, were chosen to represent three distinct canopy features. Cover represents the two-dimensional projection of the crop onto the background. Height is the three-dimensional, structural aspect of crop growth and development. Chl *a* concentration is a leaf-level measure of chemical constituents, which might be measurable because of differences in leaf

transmittance within the canopy caused by different Chl *a* concentrations.

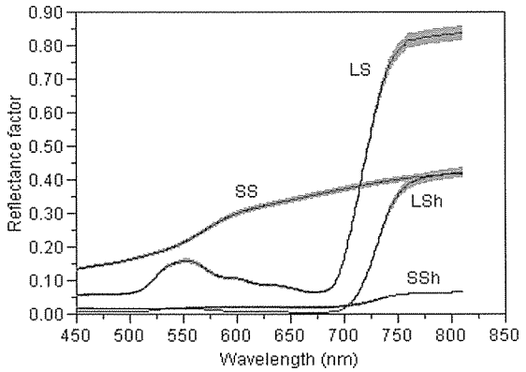

Fig. 3. Spectral references derived from ground spectroradiometer measurements used to unmix the hyperspectral imagery in the cotton component study. The central dark lines represent mean reflectance values and the shaded areas are standard error. Fraction acronoym descriptions: LS–Leaf, Sunlit; LSh–Leaf, Shadowed; SS–Soil, Sunlit (dry); SSh–Soil, Shadowed.

Results indicate that it is necessary to include the shadowed reference spectra in order to properly quantify cover, height, and Chl *a* in a canopy. Cover was well estimated by summing the LS and LSh fractions (Fig. 4a), although the Normalized Difference Vegetation Index (NDVI) performed equally well (Fitzgerald *et al.*, 2005). It was shown that height up to canopy closure could only be estimated when the LSh reference spectrum was included in the analysis (Figs. 4b, c). The Chl *a* constituent was shown to be estimated best by the LS reference alone and had a negative slope (Fig. 4d). This was due to the reference spectrum being low in chlorophyll and, thus, those spectra that matched the reference most closely (highest abundance) had the lowest Chl *a* concentrations. This was the only reference spectra combination for Chl *a* that modeled the water stressed plots well (Fitzgerald *et al.*, 2005).

CONCLUSIONS

Shadows contribute to mixed pixels and can be separated from plant and soil signals using SMA. There are two main shadow types, shadowed soils and leaves that must be included for proper unmixing. Physical components, such as cover and height were modeled well by this procedure. Chl *a* was modeled less well but choosing the appropriate reference spectrum for green leaf led to successfully including water-stressed canopy. Spider mite damage was modeled due to changes in leaf color. Future work needs to address the issues of appropriate reference (endmember) spectra to represent green plants and shadows, the minimum number of wavebands needed to define an endmember, determine endmembers stability across seasons, and the type and number of factors that can be simultaneously measured.

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Mention of specific suppliers of hardware and software is for informative purposes only and does not imply endorsement by the United States Department of Agriculture.

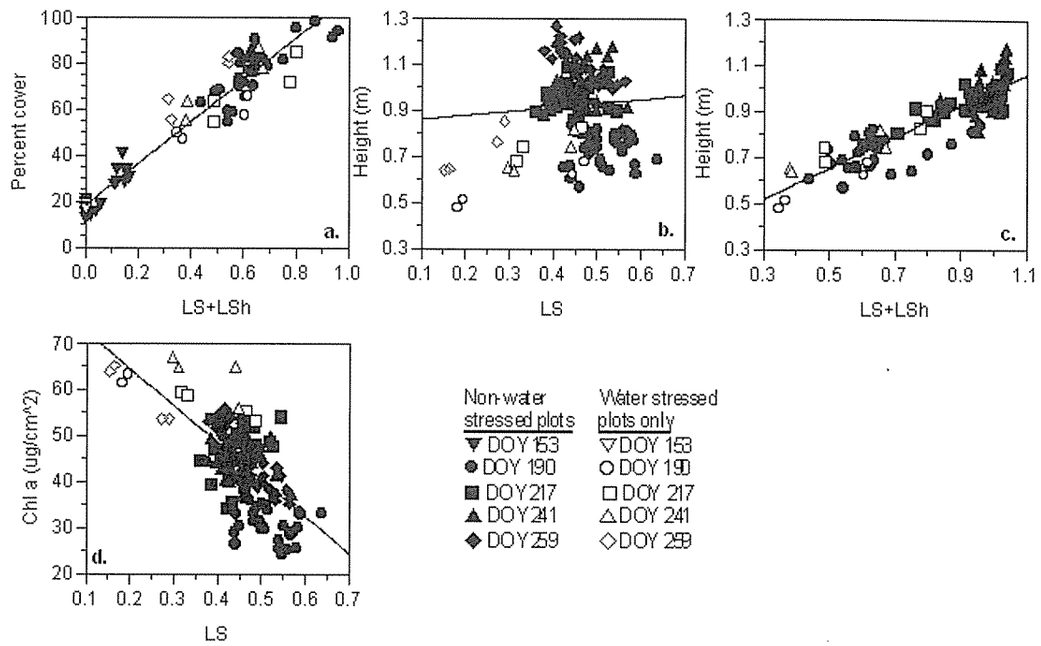

Fig. 4. Cotton canopy constituents at 5 dates in 2003 (day of year – DOY), percent cover (a), height (b, c), and chlorophyll *a* [Chl *a*] (d). Cover was modeled using sunlit (LS) and shadowed (LSh) leaf spectral components (r^2 = 0.95, RMSE = 6.6%). Height was modeled poorly by LS only but well with the shadow fraction (r^2 = 0.79, RMSE = 0.07 m). Chl *a* concentration was modeled by the LS fraction alone (r^2 = 0.46, RMSE = 7.0 µg/cm²). Open circles show water-stressed plots. All were statistically significant at α = 0.05 ($p < 0.0001$).

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Spectral Characteristics of Rice Canopy with Infestations of Leaf Folder and Brown Planthopper

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ABSTRACT

Potted experiments of rice plants (*Oryza sativa* L. cv. Tainung 67) were conducted at Taiwan Agricultural Research Institute to produce various rankings of brown planthopper [*Nilaparvata lugens* (Stål) or Homoptera:Delphacidae] or leaf folder infestations (*Cnaphalocrosis medinalis*) for canopy high-resolution spectral measurements, and then to identify spectral characteristics in associated with pest infestations leading to the establishment of remote sensing models for future applications. Reflectance spectra were taken by a field-portable spectroradiometer near ground and infestations by the pests were classified into six scales, i.e., 0 (healthy check), 1, 3, 5, 7 and 9, based on the percentages of damaged canopies. By correlation intensity analysis of spectral reflectance and infestation ranking, correlation coefficients along the measured spectral domain were determined to select spectral characteristics of the reflectance spectra. For brown planthopper infested canopies, reflectance (narrow band) at 426 nm had the highest value of correlation coefficient (r=0.878**). However, the quadratic function of single reflectance at 1450 nm improved the coefficient of determination (R^2) up to 0.857 ($P<0.01$). Among the calculated spectral indices with two spectral characteristics, six indices had R^2 greater than 0.82, especially R_{RED}/R_{NIR} ratio ($R^2=0.908$, $P<0.001$). For leaf folder infested canopies, spectral characteristics selected at near-infrared (740-1300 nm) peak (R_{NIR}) and blue light (425-490 nm) minimum (R_{BLUE}) had the highest values of R^2 (0.376 and 0.871) for measurements conducted in the tillering stage and heading stage, respectively. The reflectance difference R_{NIR}-R_{RED} in the tillering stage increased R^2 value to 0.422 ($P<0.001$), while R_{NIR}/R_{RED} ratio promoted R^2 value to 0.662 ($P<0.001$) in the heading stage, where R_{RED} is the reflectance at red light (640-740 nm) minimum. Models established by the multiple linear regression (MLR) analysis exhibited a further improvement to discriminate infestation ranking. The best six-variable MLR model improved R^2 value to 0.856 ($P<0.0001$) for the tillering stage measurements, and the best four-variable MLR model can increase R^2 value to 0.960 ($P<0.0001$) for the heading stage measurements.

Key words: Spectral characteristics, Reflectance spectrum, Rice canopy, Brown planthopper (*Nilaparvata lugens*), Leaf folder (*Cnaphalocrosis medinalis*).

INTRODUCTION

Reflectance spectrum of a plant population is known to be a function of canopy optical properties which contributed from biophysical and biochemical attributes of vegetation, viewing geometry of detector, illumination conditions of surrounding, and background effects (Asner, 1998; Barrett and Curtis, 1992; Goel, 1988; Jacquemoud *et al.*, 1992; Myneni *et al.*, 1989; Ross, 1981). The three-dimensional orientation of biophysical attributes of vegetation provides a better architecture for photons reception from incident radiation yet creates variation in the spectral characteristics. The biochemical components of plant parts bring into influences on the tissue optical nature (Baret *et al.*, 1994; Goward and Huemmrich, 1992; Jacquemoud, 1993; Kupiec and Curran, 1995). Weeds and pest infestations produce additional interference and cause difficulties in spectral discrimination between species. In fact changes of any individual or combination of elements that affect the reflection of light from a complex vegetative surface may alter the spectral pattern of vegetation.

Techniques of multi-spectral remote sensing have been applied to monitoring crop growth status for various purposes (Bauer, 1975; Bausch *et al.*, 1994; Cloutis *et al.*, 1996; Fernandez *et al.*, 1994; Shibayama and Akiyama, 1991; Walburg *et al.*, 1982; Wallace *et al.*, 1993). Due to a lack of high spectral resolution, the spectral responses of crop conditions may mix with various factors occurring at the same time (Carter, 1993; Malthus and Madeira, 1993; Tingle and Stoll, 1990), and hence limit the uses of the techniques. The development of hyperspectral remote sensing has satisfied the demand in providing qualitative and quantitative details of vegetation spectrum and in helping to assess and evaluate crop condition parameters (Blackburn, 1998; Ferns *et al.*, 1984; Yoder and Pettigrew-Crosby, 1995). In a pest infested field, the spatial variability of pest infestations obtained from analyzing hyperspectral data offers a better understanding of field conditions for site-specific pest management. The correct amount of pesticides can then be applied to the exact places at the right time. However, such applications require pre-established ground truths linkage for data verification and validation so that future practices can be directed more efficiently and economically.

Brown planthopper, *Nilaparvata lugens* (Stål), (Homoptera:Delphacidae), is considered to be a serious insect pest in many rice-producing regions including Taiwan. It is used to be the most prevalent pest island-wide bringing about destructive damage each year to rice crops (Ho and Liu, 1969; Cheng, 1978, 1990; Cheng and Lu, 1991). Rice leaf folder [*Cnaphalocrosis medinalis* Guenee (Lep., Pyralidae)], or called rice leafroller (Cheng, 1978; Miyahara, 1981; Hirao, 1982), is a widely distributed pest over Asia (Hirao, 1982)as well as a long-term major rice pest in Taiwan (Taiwan Provincial Department of Agriculture and Forestry, 1985). Leaf rolling occurs in early stages of infestation and whitish discoloration follows later. Under serious infestations, both pests cause yield reduction. If there are available information on the exact field location and levels of infestations of pest outbreaks, farmers could target pesticides applications specifically to the designated field spots where the pests are present. In this sense hyperspectral remote sensing is a suitable means to investigate spectral characteristics of rice canopy infested with pests, as the brownish and whitish discoloration appearing on the upper surface of the canopy are expected to be detected. To play an effective role in managing these pests, relationships between spectral characteristics and symptoms of infestations must be adequately investigated based on ground studies prior to the development of the management schemes.

This study took the remotely sensed high-resolution reflectance spectra of rice canopy near ground and analyzed the spectral characteristics of the infested plants. Spectral indices and regression models showing correlation with symptoms of infestation were also determined for future assessment of the plants responsive to pest infestations.

MATERIALS AND METHODS

The 8-d old seedlings of rice cultivar Tainung 67 (*Oryza sativa* L.) were transplanted to opaque plastic containers, one plant per container, with a diameter of 0.32 m and a height of 0.2 m in the first cropping seasons of 2000 and 2003. The soil was a sandy loam with a pH of 7.24. Potted plants were fertilized with ammonium sulfate at a rate of 4 g pot^{-1} as the basal dose, and two additional doses were applied within 3-wk after transplanting and 2-wk before heading, respectively, using a composite fertilizer TaiFei-39 ($N:K_2O:P_2O_5$= 12:18:12, Taiwan Fertilizer Company, Kaohsiung, Taiwan), both at a rate of 12 g pot^{-1}. Pots were kept at the experimental farm of the Chiayi Branch Station (120°27′15″E, 23°29′10″N, elevation of 35 m) of the Taiwan Agricultural Research Institute (TARI). Weeds were removed by hand-weeding and water was irrigated daily as scheduled. When reached the early grain-filling stage, 8 June 2000 and 3 June 2003, groups of pots with similar growth status and same ranking of brown planthopper infestations were selected for spectral measurements. There were 6 rankings of plant damage as that defined in Table 1. Symptoms ranged from partial to pronounced yellowing and low to high severity of stunting (INGER Genetic Resources Center, 1996). In extreme cases, symptoms observed ranged from wilting to death of plants. Reflectance spectra from years were examined and pooled for analyses.

As climatic conditions in the second cropping season are generally favorable to the infestation of rice leaf folder (Taiwan Provincial Department of Agriculture and

Table 1. Rankings of rice plant damage by the infestations of brown planthopper [*Nilaparvata lugens* (Stål)].

Ranking	Symptom
0 (CK)	No damage
1	Slight yellowing of a few plants
3	Leaves partially yellow but with no hopperburn
5	Leaves with pronounced yellowing and some stunting or wilting and 10-25% of plants with hopperburn, remaining plants severely stunted
7	More than half the plans wilting or with hopperburn, remaining plants severely stunted
9	All plant dead

Source: INGER Genetic Resources Center (1996).

Forestry, 1985), this pest has been considered a major target for pests control in the second crops in Taiwan. In the second experiment, similar cultivation practices were applied to grow rice plants in the plastic containers during the second cropping season of 2001. Dates of seedlings preparation were adjusted so that two sets of potted plants were grown to active tillering stage and heading stage, respectively. Each set of plants was infested to 6 rankings of plant damage as that listed in Table 2, and the reflectance spectra of different rankings from each set of potted plants were taken on the same day.

Table 2. Rankings of rice plant damage by the infestations of leaf folder (*Cnaphalocrosis medinalis*).

Ranking	Percentage of leaves infested (%)
0 (healthy check)	0
1	1-10
3	11-25
5	26-35
7	36-50
9	>50

Full-range (330-2600 nm) hemispherical reflectance values of targeted plants were obtained on the ground using a battery-operated field portable spectroradiometer (model GER-2600, Geophysical & Environmental Research Corp., Millbrook, New York, USA) having a 10° field-of-view (FOV) detector lens. The unit was supported by a tripod approximately 1.8 m above the canopy surface resulted in a 0.3 m diameter target area. All spectral measurements, calibrations, and graphic displays for the instrument were controlled by simple menu driven software program on a notebook computer (GER 2600 User Manual, 1996). Modes of radiance, irradiance, and percent reflectance were available for interpretation of the data. Reflectance spectrum were obtained by comparing the radiance of the target plants with the radiance of a standard reference panel, the so-called 'Spectralon' (Labsphere, Inc., North Sutton, NH, USA) for known spectral characteristics. Target and reference scans were conducted successively to produce reflectance measurements. The GER-2600 acquires measurements in 1.5 nm intervals in the visible/near-infrared (330-1050 nm) region using a 512-element silicon detector and in 11.5 nm in the wavelength range from 1050 to 2600 nm with a 128-PbS detector array. Only reflectance data in 350-2400 nm range, covering from ultraviolet to mid infrared, were used to avoid noise influences, and the band centers were rounded off to nearest whole number for application purposes (e.g., 550.22 nm as 550 nm).

At least five consistent measurements (spots) were taken from plants of each ranking and each measurement was the mean of three individual full-range spectral scans. All spectra were acquired under clear or cloudless conditions between 10:30 and 11:30 local standard time. As canopy coverage of grouped plants reached more than 90% in a nadir viewing, most parts of reflectance spectrum were found not affected by background effects by spectral comparisons. Nevertheless, care was taken to prevent the influence of shadow and background.

Changes in the spectral reflectance to the rankings of infestations were evaluated by correlation intensity analysis. With the correlation intensity curves, the intensities of linear relationship of the individual

wavebands along the analyzed spectral domain (350-2400 nm) were displayed and the spectral characteristics of interest were selected. Reflectance at three specific locations of the spectrum was also identified for narrow band comparisons and regression analysis to pest infestations. They were chosen from reflectance at the green light (490-560 nm) maximum (R_{GREEN}), the red light (640-740 nm) minimum (R_{RED}), and the near-infrared (740-1300 nm) peak (R_{NIR}). These spectral characteristics are unique in the spectrum, as the results of radiation absorption behavior of plant pigments and optical property of vegetation canopy, and are rather "dynamic" during growth, due to the influences of both internal and external factors as development in progress. Seven ration based spectral indices were also calculated for evaluating differences between rankings of pest infestations, i.e., normalized difference vegetation index (NDVI), green NDVI, soil-adjusted vegetation index (SAVI), R_{RED}-R_{NIR}, and ratios of R_{RED}/R_{NIR}, R_{GREEN}/R_{RED} and R_{NIR}/R_{RED}. The NDVI was determined by the formula $(R_{NIR}-R_{RED})/(R_{NIR}+R_{RED})$ (Elvidge and Chen, 1995) and the SAVI was computed b the formula $(R_{NIR}-R_{RED})*(1+L)/(R_{NIR}+R_{RED}+L)$, where L is defined as 0.5 in this study (Huete, 1988). The green NDVI was obtain by replacing values of R_{NIR} with the values of R_{GREEN}.

The software package Statistical Analysis System (SAS) version 8.1 (SAS, 1998) was adopted for the multiple linear regression (MLR) analysis. Reflectance of narrow bands of the reflectance spectra was used as entry into the Stepwise Selection method at the 0.15 significance level. The selected narrow bands were examined with the Collinearity Diagnostics procedure to avoid a "colliner" phenomenon to sample size. The chosen wavebands were then performed by Stepwise Selection to yield the MLR models. For instance, the best 4-variable model was in the form of $Y_4=a+a_1R_1+a_2R_2+a_3R_3+a_4R_4$, where the dependent variable Y_4 is the ranking of pest infestations and independent variable R_i is reflectance selected from chosen narrow bands of rice reflectance spectrum. The best two or more variables MLR models can be determined with a choice of user-dependent coefficient of determination (R^2) deciding the number of wavebands to be added to the models.

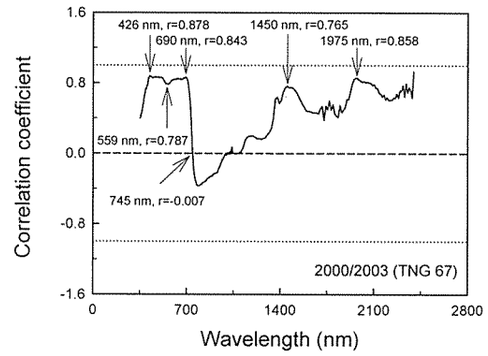

Fig. 1. Linear correlation intensity between spectral reflectance and ranking of brown planthopper infestations for rice plants grown in the early grain-filling stage of the first cropping seasons in 2000 and 2003.

RESULTS AND DISCUSSION

Linear correlation intensity between spectral reflectance and ranking of brown planthopper infestations for rice plants grown in the early grain-filling stage of the first cropping seasons in 2000 and 2003 was plotted in Fig. 1. It showed that correlation coefficient (r) varied along the wavelength

axis with the maximum absolute value located at 426 nm (r=0.878**). In a quadratic correlation, the highest value of determination coefficient (R^2) changed from 426 nm (R^2=0.793, P<0.001) to 1450 nm (R^2=0.856, P<0.001) (Fig. 2). Results indicated that spectral and optical properties and alteration of reflectance were wavelength-dependent, and variation in correlation coefficient was smaller in the visible region (350-740 nm) and higher in the infrared region (740-2400 nm), as that reported by Asner (1998).

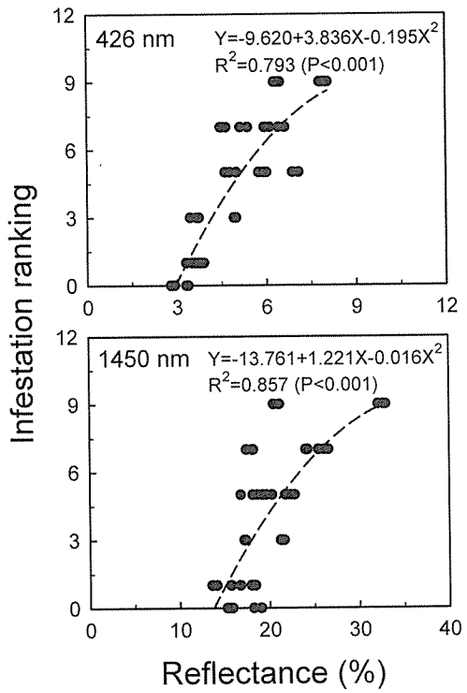

Fig. 2. The quadratic correlation between rankings of brown planthopper infestations and spectral reflectance at 426 nm and 1450 nm, respectively, for rice plants grown in the early grain-filling stage of the first cropping seasons in 2000 and 2003.

Moreover, variability of the symptoms caused by brown planthopper infestations can be accounted for up to about 86% with regression of a single spectral characteristic (1450 nm). With such a high relationship, distinct visual changes in canopy coloration (brownish) and external feature of the infested plants can be expected. The relatively stable spectral properties of rice plants, as that in other studies (Gausman, 1982; Maas and Dunlap, 1989; Walter-Shea and Norman, 1991), in the visible may be attributed to the presence of photosynthetically active pigments. These pigments have two strong absorption bands near blue (450 nm) and red (680 nm) resulting from chlorophyll activity (Salisbury and Ross, 1969). Significant increases in reflectance in the near-infrared (NIR) are due to photon scattering at the air-cell interfaces within the leaf spongy mesophyll (Woolley, 1971; Boyer *et al.*, 1988). The NIR also exhibits the strongest multiple-scattering in the green foliage canopy because of high reflectance plus transmittance of foliage in this region (Asner, 1998; Barrett and Curtis, 1992).

By using spectral indices incorporating with two spectral characteristics to correlate with infestation rankings, R^2 was improved in most indices, especially the exponential function of R_{NIR}/R_{RED} ratio (R^2=0.922, P<0.001) (Fig. 3). In a linear relationship between infestation ranking and value of spectral index, R^2 may increase to 0.908 (P<0.001) using the R_{RED}/R_{NIR} ratio as inputs. Results further suggest that infestations of brown plant hopper would affect the factors involved in pigment absorption (responsive to alteration of R_{RED}) and the vegetation coverage (responsible for fluctuation of R_{NIR}). Usually, reflectance in the green band increases markedly following

the degradation of chlorophyll, while reflectance decreases substantially across the whole range of infrared wavebands due to the changes in the internal structure (Barrett and Curtis, 1992). Accordingly, similar phenomena with respect to the increment in visible reflectance and the reduction in infrared reflectance might occur in rice plants infested with brown planthopper, as that reported in plants infected by a disease (Colwell, 1969; Leeman *et al.*, 1971) and in dehydrated conditions (Aldakheel and Danson, 1997; Rollin and Milton, 1998).

In case of leaf folder-infested rice, pattern of linear correlation intensity was not similar for plants grown in the active tillering stage and in the heading stage (Fig. 4). The linear relationship between spectral reflectance and infestation ranking was negative and was more closely correlated in

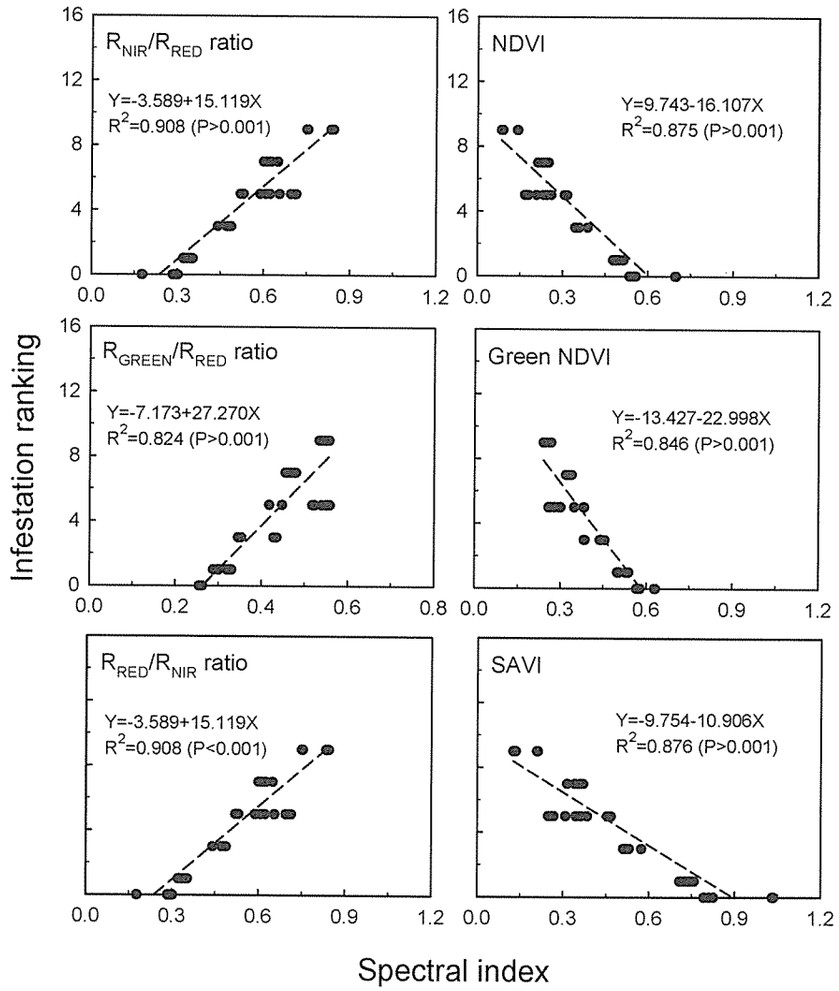

Fig. 3. Changes of infestation rankings to spectral indices in plants of brown planthopper-infested rice grown in the early grain-filling stage of the first cropping seasons in 2000 and 2003.

the heading (r=-0.493 to r=-0.928) than in the active tillering (r=-0.0973 to r=-0.613). The maximum absolute value of correlation coefficient located at 757 nm (r=0.613*) in the active tillering but shifted to 445 nm (r=0.928**) in the heading. The relation of infestation ranking to reflectance was best fitted to a linear function at 757 nm (R^2=0.376, P<0.001) in the active tillering stage yet was best fitted to a quadratic function at 445 nm (R^2=0.881, P<0.001) in the heading stage (Fig. 5). Apparently, the whitish color and leaf rolling symptoms appeared on the vegetative tissues of infested plants did not strongly link to reflectance performance as those shown in the heading period.

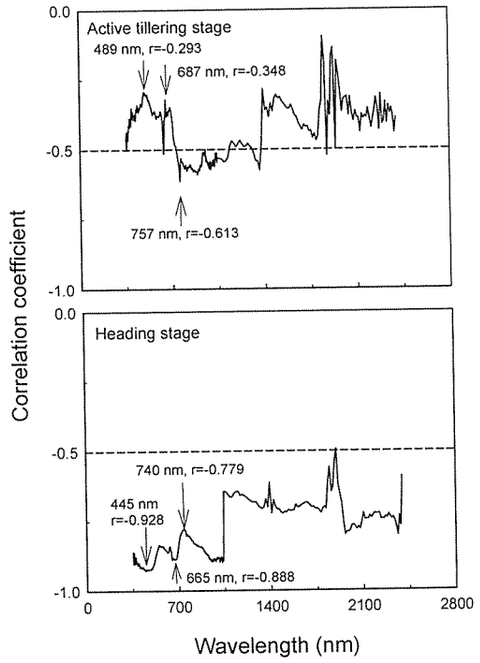

Fig. 4. Linear correlation intensity between spectral reflectance and ranking of leaf folder infestations for rice plants grown in the active tillering and the heading stages of the second cropping season in 2001.

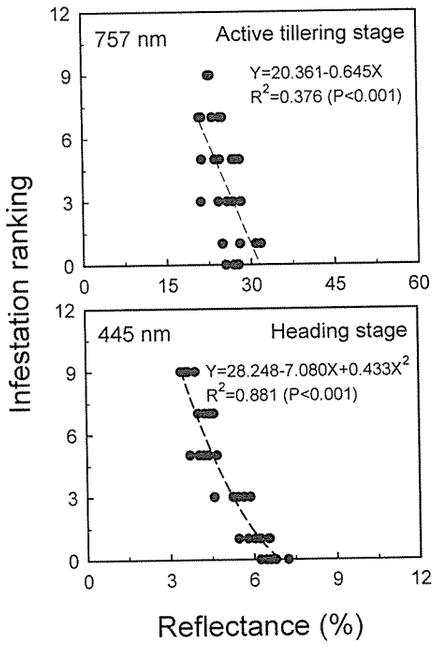

Fig. 5. The relationships between rankings of leaf folder infestations and spectral reflectance at 757 nm and 445 nm for rice plants grown in the active tillering and the heading stages, respectively, of the second cropping season in 2001.

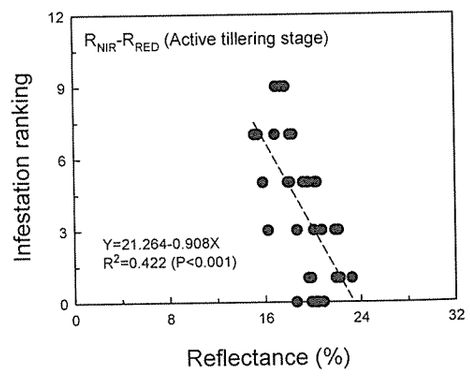

Fig. 6. Changes of infestation rankings to spectral index (R_{NIR}-R_{RED}) in plants of leaf folder-infested rice grown in the active tillering stage of the second cropping season in 2001.

When differentiating rankings of severity with spectral indices, only the reflectance difference between R_{NIR} and R_{RED} was significantly linearly correlated (R^2=0.422, P<0.001) with the infestation ranking in the active tillering stage (Fig. 6). There was a slight increase (0.376 to 0.422) in R^2 from a single spectral characteristic to a two-characteristic index. However, no increase in R^2 was found in the examined spectral indices in the heading stage (Fig. 7). Therefore, the spectral indices calculated by the spectral characteristics R_{GREEN}, R_{NIR} and R_{NIR} have no improvement for the evaluation of rice leaf folder infestations.

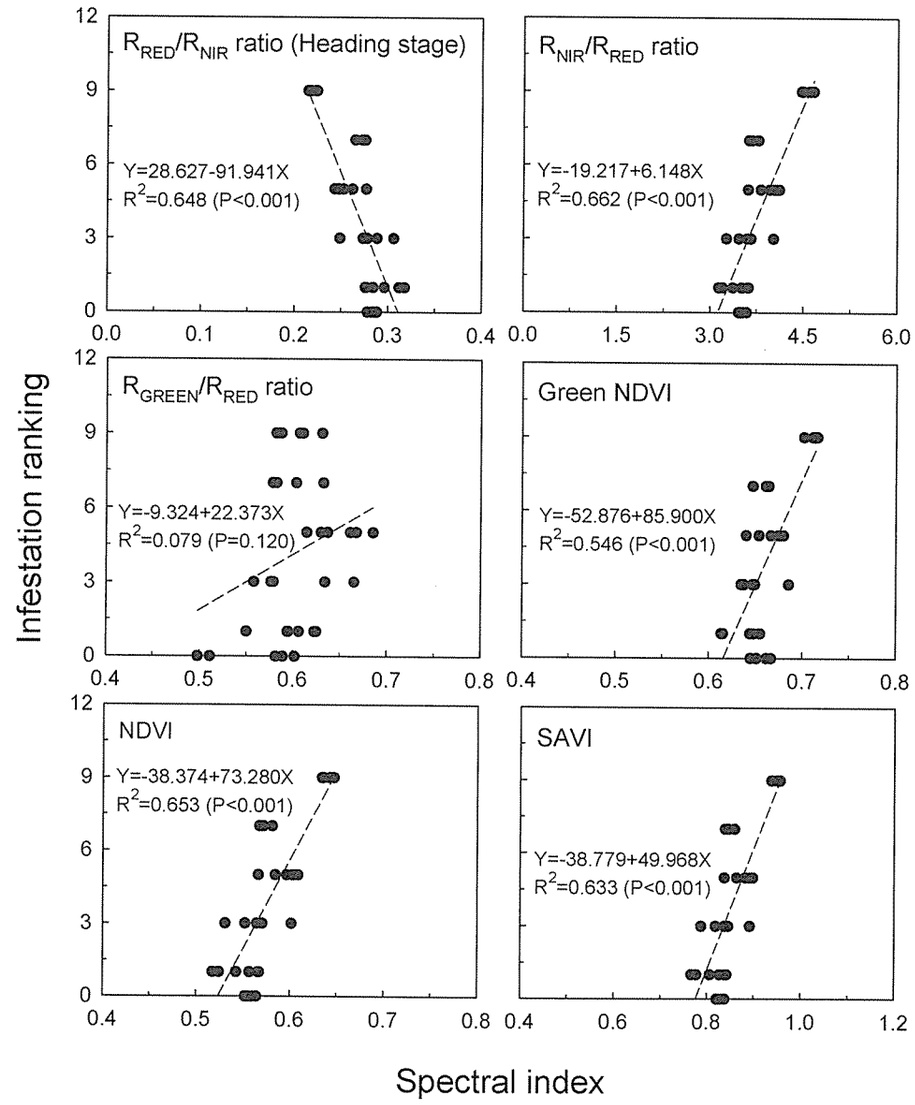

Fig. 7. Changes of infestation rankings to spectral indices in plants of leaf folder-infested rice grown in the heading stage of the second cropping season in 2001.

Table 3. The best six-variable and four-variable linear regression models for evaluation of leaf folder (*Cnaphalocrosis medinalis*) infestations for rice plants (*Oryza sativa* L. cv. TNG 67) grown in the active tillering and the heading stages, respectively, in the second cropping season of 2001.

	$Y_4 = a + a_1R_{\lambda1} + a_2R_{\lambda2} + a_3R_{\lambda3} + a_4R_{\lambda4} + a_5R_{\lambda5} + a_6R_{\lambda6}$									
Growth stage	a	a_1	a_2	a_3	a_4	a_5	a_6	R^2	P	$C_{(P)}$
Active tillering†	27.795	-2.033	-0.924	-0.511	5.338	-2.150	-3.447	0.856	<0.0001	8.37
Heading‡	12.711	-8.722	-1.060	0.757	7.327			0.960	<0.0001	5.00

† The best six-variable linear regression model: $R_{\lambda1}$, reflectance at 757 nm; $R_{\lambda2}$, reflectance at 1846 nm; $R_{\lambda3}$, reflectance at 1937 nm; $R_{\lambda4}$ reflectance at 1727 nm; $R_{\lambda5}$, reflectance at 1963 nm; $R_{\lambda6}$, reflectance at 1686 nm.

‡ The best four-variable linear regression model: $R_{\lambda1}$, reflectance at 517 nm; $R_{\lambda2}$, reflectance at 974 nm; $R_{\lambda3}$, reflectance at 929 nm; $R_{\lambda4}$, reflectance at 541 nm.

The impact of leaf folder infestations on the spectral reflectance of rice plants grown in the active tillering and the heading stages of the second cropping season in 2001 was further evaluated by using the MLR approach. Reflectance of narrow bands from the measured spectral domain of each infestation rankings were all used as inputs for MLR analyses. Results showed that with reflectance of six narrow bands as input parameters, R^2 was promoted to 0.856 ($P<0.0001$) in the active tillering stage, while R^2 was improved to 0.960 ($P<0.0001$) with four parameters in the heading stage (Table 3). Obviously the MLR models combining spectral reflectance from more than two wavebands not only provided flexibility in choosing the individual narrow bands (Yang and Chen, 2004) but also exhibited a greater sensitivity to phenological variation of leaf folder–infested plants and improved the models' ability to estimate pest infestations.

As observed in nature and in this study, discoloration of leaves is one of the first indications of pest occurrence in rice plant. When plants are suffering from pests attacks, there are changes in leaf structure and color, providing a good indication of pest infestation. Results further indicate that spectral bands across the visible and infrared region are useful for monitoring differences in the conditions of rice infested by brown planthoppers and leaf flolder. The spectral characteristics selected in this study have shown their potential in discriminating the rankings of pest infestations. In practical applications, however, the filed spectral response remotely sensed from air-borne and space-borne platforms may be rather different than those from the ground truth. Further studies are required to link the gaps between ground-based remote sensing and real world conditions. Besides, such relationships may vary qualitatively and quantitatively during the growing season as plants age and may be influenced by the conditions of the atmosphere and the viewing geometry of detectors (Asner, 1998; Goel, 1988; Gerstl, 1990; Myneni and Asrar, 1993; Meyer *et al.*, 1995).

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Oral Session 4

Identification of *Bacillus* spp., *Escherichia coli*., *Salmonella* spp., *Staphylococcus* spp. and *Vibrio* spp. with 16S Ribosomal DNA Based Oligonucleotide Array Hybridization

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ABSTRACT

Rapid identification of the genus and species of bacteria in foods and clinical specimens is important. In this report, DNA sequences of bacterial 16S rDNA were used to develop the oligonucleotide array for the identification of bacterial strains of *Bacillus* spp., *Escherichia coli*, *Salmonella* spp., *Staphylococcus* spp. and *Vibrio* spp. Most of these bacterial strains may cause food-borne outbreaks or sporadic cases. A rapid (<4 h) detection method that used universal PCR primers to amplify the variable regions of bacterial 16S rDNA, followed by reverse hybridization of the PCR products, which were biotin labeled, to the oligonucleotides arrayed on the chip was developed. Fifteen oligonucleotide probes were selected and spotted on the nylon strip to determine the array hybridization patterns. It was successful in discriminating *Bacillus* spp., *Escherichia coli*, *Salmonella* spp., *Staphylococcus* spp. and *Vibrio* spp. with identification, in general, to the genus level, not species level. As 182 randomly selected strains were assayed, the detection rate was found higher than 98%. Except for 3 strains, the remaining 179 strains were correctly identified and no cross reactions were observed. These 179 strains generated five hybridization patterns. Adding more oligonucleotide probes to the array may allow the detection of more bacterial genera and species without significant increasing the complexity or cost.

Key words: 16S ribosomal DNA, Oligonucleotide array, Food pathogen.

INTRODUCTION

Bacterial identification methods currently used are based on the analysis of morphological, physiological, biochemical, and genetic data of the target bacteria. In the last decade, rapid diagnostic methods have been developed by direct detection of the specific genes of bacteria in clinical and food samples, and many species or genus specific oligonucleotides have been developed for such detection (Laberge *et al.*, 1996; Newcombe *et al.*, 1996; Hsu and Tsen., 2001; Chizhikov *et al.*, 2001; Tsen and Chen., 2001; Rudi *et al.*, 2000). These systems are usually used to confirm the diagnosis of specific clinical syndromes or specific bacterial strains (Chun *et al.*, 1999; Daffonchio *et al.*, 1998; Tsen *et al.*, 1999). These results suggest that DNA based methods may be more sensitive and rapid than the conventional bacteriological methods.

However, the use of different primers for various bacterial strains is impractical for the routine analysis of food or clinical samples that may contain one or more of many possible pathogens. For such specimens, either a complex PCR with a mixture of large numbers of primers is needed, or a large series of individual PCRs must be run in parallel, or sequentially. Multiplex PCR may be effective for a limited number of organisms, but as more primers are added, the detection sensitivity for some target bacteria decreases and the chance that two unrelated primers produce spurious product increases (Tsen *et al.*, 2001). These problems can be solved by using a biochip arrayed with oligonucleotides specific for different bacteria species.

Recently, 16S rRNA gene (*rrn*) operon has been used for the designing of DNA probes and PCR primers specific for some bacteria genera and well-solved species, such as *E. coli* (Tsen *et al.*, 1998), *Listeria monocytogenes*, *Salmonella* spp. (Gendel *et al.*, 1996; Lin *et al.*, 1996; Tsen *et al.*, 1999), and *Bacillus cereus* group species, including *B. cereus*, *B. thuringiensis*, *B. anthracis*, and *B. mycoides* (Ash *et al.*, 1991; Giffel et al., 1997). Genetic variations in 16S rDNA are intergeneus and interspecies for some well studied species and this variation could be utilized for the designing of oligonucleotides used in array identification system. Bacteria could be identified directly by amplification of bacterial 16S rDNA, 23S rDNA or the intergenic spacer region (ISRs), followed by reverse hybridization of the labeled amplified product to an oligonucleotide array designed to differentiate the sequence variation of the bacteria. With such a principle, Anthony et al. (2000) has developed a 23S rDNA based oligonucleotide array system for the rapid diagnosis of bacteremia. Also, a very recent report showed that using 16S rDNA PCR and DNA array, 15 fish pathogens might be simultaneously discriminated (Warsen *et al.*, 2004).

In this report, by using an array of specific oligonucleotides designed from the variable regions of 16S rDNA sequences, and the reverse hybridization of 16S rDNA amplified products to this array, we established the specific hybridization patterns for strains of *Bacillus* spp., *Escherichia coli*, *Salmonella* spp., *Staphylococcus* spp., and *Vibrio* spp.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains used in this study are listed in Table 1. These strains were obtained from American Type Culture Collection (ATCC, Rockville, MD, U.S.A.); Culture Collection and Research Center (CCRC, Hsin-Chu, Taiwan); National Pingtung University (PT, Pingtung, Taiwan); United State Department of Agriculture (USDA, Washington D.C., USA) and Taiwan Agriculture Chemical and Toxic substances Research Institute (TACTRI, Taichung, Taiwan).

Table 1. Bacterial strains and their oligonucleotide array hybridization patterns.

Strains	Pattern	No. of strains	Strain no. and sources
B. cereus group	C	23	*B. cereus* ATCC 14579, 13061, 21182, 21281; CCRC 10250, 10282, 10603; Bc16-31 (this lab).
		30	*B. thuringiensis* ATCC 33679, 10729; CCRC 11501, 14377, 15070;Tt01~25 (TACTRI).
		3	*B. mycoides* ATCC 6462, 10206; CCRC11968.
		2	*B. anthracis* ATCC 8705, 14578.
Other *Bacillus* spp.	C	9	*B. subtilis* (CCRC 10029, 10258, 10267, 10447); *B. polymyxa* (ATCC 842); *B. coagulans* (CCRC 10251); *B. firmus* (ATCC 14575); *B. pumilus* (ATCC7061); *B. lentus* (ATCC10804).
E. coli	A	25	ATCC 11775, 25922, 43886 (ETEC), 43894 (EHEC); CCRC 12570, 14824 (EHEC), 13086 (EHEC), 41443; WHO 110 (ETEC); E01~08; E2418~2425 (this lab).
Salmonella spp.	B	5	*S. enterotidis* ATCC 13076; PT 633,661; SE06, 07 (this lab).
		7	*S. typhi* CCRC 14875; ATCC 8427; ST01-04, 40 (this lab).
		10	*S. typhimurium* ATCC 14028, 13311; STM01-03, ST604, ST62, 63, 80, 90 (this lab).
		32	*S. aba; S. anatum; S. arkansas; S. azteca; S. bareilly; S. berta; S. borismorbificans; S. braenderup; S. choleraesuis; S. cerro; S. coleypark; S. colindale; S. derby; S. dublin; S. emek; S. eppendrof; S. florida; S. goerlitz; S. hadar; S. havana; S. infantis; S. meleagridis; S. memhaden; S. montevideo; S. muenchen; S. newport; S. oranienburg; S. panama; S. richmond; S. saintpaul; S. victoria; S. worthington.*
Staphylococcus spp.	D	10	*Staph. aureus* CCRC 12653, 12654, 12656, 12657, 12660, 13831; STA 0029, 0040, 0042, 0043 (this lab.).
		5	*Staphy. epidermidis* (ATCC 155); *Staphy. haemolyticus* (ATCC 29970); *Staphy. hominis* (ATCC 27844); *Staphy. simulans* (ATCC 11631); *Staphy. warneri* (ATCC 27836).
Vibrio spp.	E	10	*V. parahaemolyticus* CCRC 12863, 12963, 13023, 13025; VP 1253, 1273, 1287, 1311, 1328, 1354 (this lab.).
		11	*V. alginolyticus; V. cholerae; V. cincinnatiensis; V. fluvialis; V. furnissii; V. harveyi; V. mediterranei; V. mimicus; V. proteolyticus; V. tubiashii; V. vulnificus.*
Other bacteria spp.		4	*Pseudomonas aeruginosa* (ATCC 10145), *Latobacillus brevis* (CCRC 12187), *Proteus vulgaris* (CCRC 10728), *Streptococcus faecalis* (CCRC 10066)

PCR primers for 16S rDNA

The primer sequences were chosen from the conserved regions previously reported for the bacterial 16S rDNA. Partial 16S rDNA sequences flanking variable regions (V) from V1 to V3 were used. The PCR primers were designed as follows: forward primer, 16S-350F and reverse primer, 16S-820R. Primers were synthesized with a model 394 DNA-RNA synthesizer (Applied Biosystems, Foster City, Calif.).

DNA isolation and PCR amplification

Genomic DNA was extracted and purified using Genomic Tips (Qiagen, Valencia, Calif.) with Genomic DNA Buffer Set (Qiagen). The concentration of DNA was determined by UV spectrophotometry and with SpotCheck (Sigma, St. Louis, Mo.). 16S rDNA fragment was amplified by using the primer set 16S-350F and 16S-820R that were targeted to universally conserved regions. The amplification reactions were performed with a GeneAmp 2400 PCR thermocycler (Perkin-Elmer, Norwalk, Conn.). The PCR products were confirmed by 2% agarose gel electrophoresis. For biotin labeled PCR product preparation, similar conditions were used except that the primers used were 5'-biotin labeled 16S-350F and 16S-820R. These biotin primers were synthesized by MDBio Inc. (Taipei, Taiwan).

Oligonucleotide synthesis and array fabrication

BLAST search was used to find and retrieve the sequences homology for each 16S rRNA genes. Included in the procedure were 16S rDNA sequences (with accession number) of *Salmonella enteritidis* (U90318), *Vibrio parahaemolyticus* (AB016271), *Salmonella typhi* (U88545), *Escherichia coli* (J01859), *Salmonella typhimurium* (U90316, U88546), *Staphylococcus aureus* (AF146368), and *Bacillus cereus* (Z84576). Using the CLUSTAL X Multiple Sequence Alignment Program (Strasburg, France), nucleotide sequences of the bacterial 16S rDNA obtained from the Gene Bank database were aligned and the oligonucleotides (Table 2) could be designed using the ArrayDesigner software (PREMIER Biosoft, CA, USA.). Oligonucleotides purified from C18 Nucleosil column (Sigma, St. Louis, Mo.) were melted in TE buffer and spotted onto the nylon membrane (Hybond-N+, Amersham, USA.) by using the Manual Glass Slide Indexing System (VP 470 and 478A, V&P SCIENTIFIC Inc., CA, USA.). Each spot containing 5 ng oligonucleotide DNA. The spotted DNA was then cross linked onto to the nylon membranes by UV irradiation with CROSSLINKER CL-508 (UVItec, Cambridge, ENGLAND) at 0.12 joule/cm^2. The membranes were stored at 4 ℃ before use.

Hybridization and chromogenic reaction

The biotin-labeled 16S rDNA PCR amplicons were hybridized to the oligonucleotides arrays using the following protocol. Each membrane strip was numbered and placed in a separate 1.5-ml screw-top microcentrifuge tube containing 1.0 ml of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% N-laurylsarcosine, 0.05% SDS, 10 μg of heterologous salmon sperm DNA (Sigma Chemical Co.), and 1% blocking reagent (Roche, IN., USA). The biotin labeled PCR products were heated to 99℃ for 3 min and cooled to 4 ℃ immediately in the PCR thermal cycler, followed by transferring to

each proper hybridization tube. Hybridization was carried out for 60 min at 50℃ with gentle agitation.The membrane strips were removed from the tubes and washed twice with 5 ml 0.1× SSC-0.1% SDS at 37℃ for 5 min. They were then blocked with 2 ml of blocking reagent (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.5% skim milk) for 15 min followed by incubation for 30 min with the same buffer containing 1/1000 volume of streptavidin-alkaline phosphatase (NEN, Boston, MA, USA.). Afterwards, the strips were washed twice in 5 ml Tris-buffered saline (TBS, pH 7.4) followed by chromogenic reaction. For this reaction, the membrane strips were immersed into 1 ml 5-bromo-4-chloro -3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT) substrate solution (NEN, Boston, MA, USA.) with gentle agitation, and the color developed could be clearly observed after 10 to 30 min. After drying the strips at 65℃ for 5 min, the relative signal intensities were determined by scanning the membranes with an UMAX Power Look 3000 (UMAX, Texas, USA.). The results were analyzed by using the Gel-Pro ANALYZER software (Media Cybernetics, Silver Spring, Md. USA).

RESULTS

Design of PCR primers and fabrication of oligonucleotide array

To construct the oligonucleotide array for the detection of food-borne pathogen, the conserved regions flanking variable region 1 (V1) to 3 (V3) of the bacterial 16S rDNA (Fig. 1) were used to design the two consensus primers, 16S-350F and 16S-820R. Using the 5′ end biotin-labeled primers from 16S-350F and 16S-820R, DNA sequence flanking the V1 to V3 regions of the 16S rRNA genes for the bacterial strains to be detected could be amplified. Molecular weights of these PCR products were equal to 475 bp (Fig. 2). Target bacterial strains selected included strains of common food pathogens that may cause diarrhea or vomiting, strains of *B. thuringiensis*, which may produce enterotoxigenic activity (Yang *et al.*, 2003), and other bacterial strains (Table 1). Under the PCR conditions described in Methods, only very few strains, i.e., 3 of the total 182 strains assayed, could not generate any amplification products. For example, three strains, i.e., *Bacillus spirit*, *B. narit* and *Salmonella* Vento, gave negative PCR results. Lowering the stringent conditions may enhance the PCR for these strains.The oligonucleotides probes to be arrayed were obtained by use of the software Array-Designer (CA, USA.) (Table 2). All the bacterial 16S rDNA sequences available in NCBI Gene Bank database, were collected. Initially, 36 oligonucleotides were selected. PCR amplicons obtained from amplification of the bacteria chromosomal DNAs using consensus primers were then hybridized to the array. Most of the hybridization results were in agreement with those predicted from

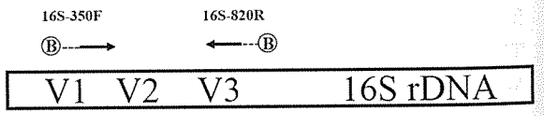

Fig. 1. Relative positions of the universal PCR primers. Both primers were labeled with biotin at the 5′end and after the PCR product was denatured, it served as probes to hybridize with the oligonucleotides arrayed on the nylon membrane.

Table 2. Oligonucleotide probes and PCR primers used in this work.

Oligonucleotides (dots) [a]	Sources[b]	Accession Number
No. 1	*B. cereus* (anti-sense)	Z84576
No. 2	*B. cereus* (anti-sense)	Z84576
No. 3	*B. cereus*	Z84576
No. 4	*B. cereus* (anti-sense)	Z84576
No. 5	*B. cereus*	Z84576
No. 6	*Staph. aureus*	AF146368
No. 7	*B. cereus (anti-sense)*	Z84576
No. 8	*E. coli*	J01859
No. 9	*B. cereus*	Z84576
No. 10	*Staph. aureus*	AF146368
No. 11	*E. coli*	J01859
No. 12	*V. parahaemolyticus*	AB016271
No. 13 [c]	*E. coli (forward primer)*	J01859
No. 14	*E. coli*	J01859
No. 15	*E. coli*	J01859
No. 16	*Salm. typhimurium*	U90316, U88546
16S-820R [c]	*E. coli (reverse primer)*	J01859

[a] Oligonucleotides No.1-16 were spotted onto the nylon membranes.

[b] 16S rDNA sequence from the bacteria shown was aligned with those of other bacteria and thus the oligonucleotides were designed.

[c] The universal forward (16S-350F) and reverse (16S-820R) primers were biotin labeled on the 5′ end.

the probe sequences. Each PCR product hybridized to its corresponding specific oligonucleotide probe, but some PCR amplicons cross hybridized with other tested oligonucleotide probes. For example, PCR amplicons generated from *Salmonella* spp. hybridized with No. 15 oligonucleotide probe which was deduced from *E. coli* 16S rDNA. Also, PCR amplicons from *Vibrio* spp. hybridized not only with its own specific oligonucleotide No. 12, but also with oligonucleotide probes No. 8 and 15. Probes No. 8 and 15 were both deduced from the *E. coli* 16S rDNA (Table 2). Some of the designed oligonucleotide probes were eliminated from the membrane strip, unless they were useful for the pattern differentiation. Also, oligonucleotide probes that could not hybridize with any bacterial PCR amplicons were excluded from the array. After spotting the useful fifteen oligonucleotide probes on the membrane strip followed by hybridization with biotin labeled PCR products prepared from bacterial strains shown in Table 1, five hybridization patterns (A → E) (Fig. 3) corresponding to five bacteria genera, i.e., *E. coli*, *Salmonella* spp., *Bacillus* spp., *Staphylococcus* spp. and *Vibrio* spp., (Table 1, Fig. 3) were obtained.Due to the fact that oligonucleotide probe No. 11 could hybridize with amplicons from all the 179 bacterial strains tested plus some other unlisted strains (data not shown), it was thus used as the probe for positive control. Also, probe No. 13, equivalent to biotin-labeled primer 16S-350F, serve as a positive control for the chromogenic reaction.

Different parameters for array hybridization, such as temperature, incubation time, salt composition and shaking speed, were optimized for both the specificity and sensitivity of the array system. The optimized conditions were given in the experimental methods. For hybridization patterns, all the *E. coli* strains tested were able to hybridize with probes No. 8, 11, 14 and 15 and generate a specific pattern A. From the patterns shown in Fig. 3, it may be concluded that oligonucleotide No. 14 is a species specific probe for *E. coli*, although strains of the closely related genus *Shigella* also generate the same pattern (data not shown). Also, oligonucleotide No. 16 and No. 12 are the genus specific probes for *Salmonella* spp. and *Vibrio* spp., respectively. *Salmonella* and *Vibrio* spp. generated a specific oligonucleotide array hybridization patterns B and E, respectively (Fig. 3). Meanwhile, probes No. 4, 5, 6, 7, 9, 10 and 11 were the co-shared probes for strains of *Bacillus* spp. and *Staphylococcus* spp., and probes No. 1-3 were genus specific probes for strains of *Bacillus* spp. Almost all the *Bacillus* strains tested produced the specific hybridization pattern C. For *Staphylococcus* spp., all the *Staphylococcus* strains tested generated hybridization pattern D, from which, however, specific and unique probes for *Staphylococcus* spp. could not be found.

In order to confirm the hybridization patterns shown in Fig. 3, all the 182 isolates belonging to the five genera were analyzed and 179 strains generated the expected hybridization patterns (Table 1). The 3 strains showing negative hybridization results could not generate any PCR amplicon with the consensus primers.

Sensitivity and specificity

Under the experimental conditions, PCR products amplified from 1~5 X 10^3 cfu/ml of the cell dilutions could be observed when the products were electrophoresed with 2% agarose gel (Fig. 2). In some cases, the PCR product was not visualized on the ethidium bromide stained agarose gel, however, after hybridization of the PCR products to the oligonucleotide array, hybridization signals were visualized. Thus, PCR method followed by hybridization of the products to the oligonucleotide array would improve the detection sensitivity. For the bacterial strains tested, the detection limit for *E. coli* and *V. parahaemolyticus* (500 cfu / ml) was the lowest, followed by *Salmonella* spp. On the other hand, *Staphylococcus* spp. and *Bacillus* spp., gave higher detection limits, i.e., about 4,500 cfu/ml. Chromosomal DNA from *Pseudomonas aeruginosa* (ATCC 10145), *Latobacillus brevis* (CCRC 12187), *Proteus vulgaris* (CCRC 10728), *Streptococcus faecalis* (CCRC 10066) could produce proper PCR amplicons with the consensus primers but not the visible array hybridization signals.

To further confirm the identification results, some genus specific oligonucleotide probes were selected and arrayed in three repeats onto new strips (Fig. 4). For such analysis, all the target strains could produce expected hybridization patterns. For example, all the 58 *Bacillus* strains assayed hybridized with oligonucleotide No. 1 and No. 2, and produced pattern "a" in Fig. 4. All the strains of other bacteria spp; i.e., *E. coli*, *Salmonella* spp. and *Vibrio* spp. produced their own specific hybridization patterns, i.e., pattern b, c, d, respectively, on such new arrayed strips (Fig. 4). These results demonstrate that these five selected oligonucleotide probes could serve as genus specific probes.

DISCUSSION

For simultaneous detection of two or more bacterial pathogens, multiplex PCR with several sets of species-specific primers could be used (Tsen *et al.*, 1999; Lin *et al.*, 1996). However, in some cases, different primer sets may generate PCR products with molecular weights similar or closer to each other. Also, mixture of several primer sets might lead to poor amplification efficiency, and lowering the annealing temperature might generate false-positive results. Modification of the multiplex PCR conditions may not be able to eliminate the interference caused by false-positive bands (Wilson *et al.*, 2002). The method described in the present study resolves these problems. Only a single running of PCR with a single set of primers derived from the conserved sequences of a common gene is needed.

The common gene used for the designing of oligonucleotide array is 16S rDNA since it is generally accepted that the sequence of 16S rRNA can be used to distinguish genera and well resolved species (Plays *et al.*, 1997). However, the rRNA (*rrn*) operons are often multigenic in bacteria genome with the number of *rrn* operons ranging from 1 to 15 (Acinas *et al.*, 2004). For example, both *Salmonella* spp. and *E. coli* contain seven *rrn* operons (Condon *et al.*, 1995). Thus, oligonucleotide probes or primers designed from the bacterial 16S rDNA sequence retrieved from the database in Genebank may not allow the detection of all strains in a bacteria species (Lin *et al.*, 2004). It should be mentioned that sequence variation between 16S rDNA copies is usually < 1%, however, this may be greater between strains of the same species depending on the region being used as a probe.

It is sometimes difficult to find suitable signature sites on the 16S rRNA for the identification of bacteria on the species level.

In this regard, the 23S rRNA with approximately 3,000 nucleotides has twice as many potential probe target sites as the 16S rRNA (Fuchs *et al.*, 2001). However, there are not many 23S rRNA sequences in the databases, compared to more than 18,000 16S rRNA sequences (Maidak *et al.*, 2000). Also, by the use of longer probes, it is consequently not helpful for fast and unambiguous identification of closely related species in the DNA array hybridization reaction (Hedegaard *et al.*, 1999). Using the

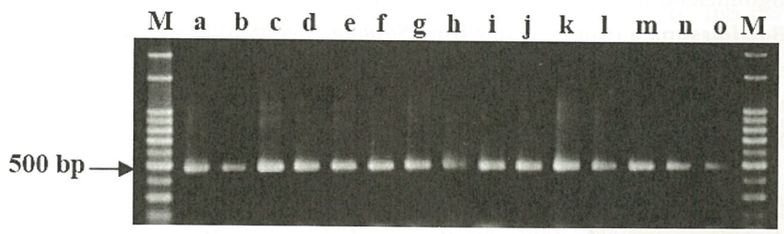

Fig. 2. PCR products amplified with the universal 16S rDNAPCR primers. PCR products amplified from different strains, i.e., lanes: a-e, *E. coli*ATCC 11775, 25922, 43894, 43894, CCRC 12570; f-j, *B. thuringiensis*ATCC 33679, 10729, CCRC 11501, 14377, 15070; k-o, *S. aureus*CCRC 12653, 12654, 12656, 12657 and 12660, are shown. Lane M represent the 100-bp DNA ladder.

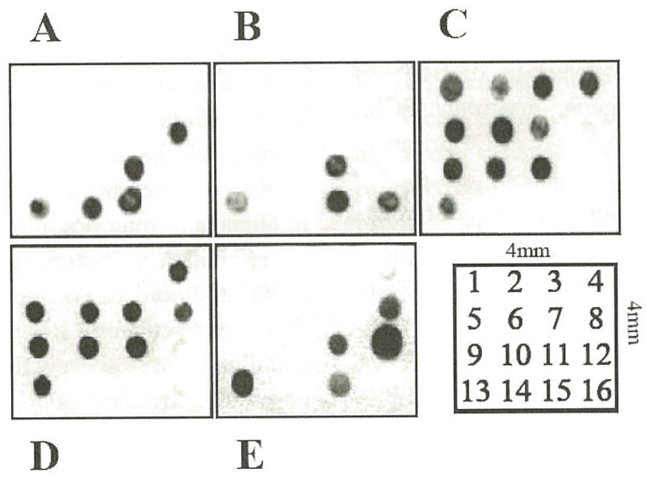

Fig. 3. Bacteria hybridization patterns using the 16S rDNAoligonucleotide array. The patterns are Afrom *Escherichia coli*; Bfrom *Salmonella*spp.; Cfrom *Bacillus*spp.; Dfrom *Staphylococcus* spp.and Efrom *Vibrio* spp. The numbers of the oligonucleotides (Table 1.) spotted on the membrane were indicated in the lower right-hand strip. Dot No. 13 was the biotin labeled forward primer which served as the chromogen development positive control.

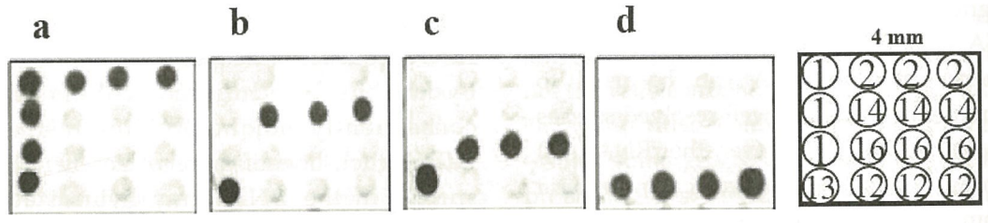

Fig. 4. The oligonucleotide three repeats verification. The hybridization patterns obtained are a from *Bacillus*spp.; b from *E. coli*; c from *Salmonella*spp.; d from *Vibrio*spp. The numbers of oligonucleotides spotted on the membrane were indicated on the strip. These oligonucleotides were those of No.1, 2, 12, 14 and 16, corresponded to those in Table 2. Dot No. 13 served as a chromogenic positive control.

23S rDNA based array system, when 158 detected, only 125 (79.7%) were correctly identified (Anthony *et al.*, 2000). Although a 16S rDNA based microarray has been developed for the discrimination of 15 fish pathogens, for each bacteria species, only one strain was tested (Warsen *et al.*, 2004). In unselected positive blood culture broths was their report, Warsen *et al* (2004) pointed out that it is possible that a strain that should be identified using such microarray might be missed due to a random mutation within this region and thus produced a false negative. They do not know what the frequency of

these events might be at a population level. As described earlier, the rRNA gene are multigenic and the rDNA sequences they retrieved form the Genebank for oligonucleotides designing may represent only one rrn operon, which may not exist in some strains of the same species. Warsen *et al* (2004) also pointed out that an additional challenge with their strategy is that two different species may share the same sequence identity within the probe region (Fox *et al.*, 1992). In fact, 16S rRNA sequence identity may not be sufficient to guarantee species identity (Fox *et al.*, 1992, Plays *et al.*, 1997). Although the microarray shown in this report allows the discrimination of bacterial strains mainly on the genus level, the detection rate for bacterial strains was higher than 98.3%. This was shown by the assay of 182 strains shown in Table 1.

An ideal oligonucleotide should hybridize only to one bacterial species and to all members of the strains within that species. However, in this study, we found that probes sequences with homology higher than 85% to bacteria other than target strain may cross react with strains of these closely related species. In such cases, two or more additional oligonucleotides may be required to allow the discrimination of the bacterial strains (Fig. 3, pattern C, D). Using the array system, the clonal relationship between bacterial strains might be elucidated. For example, *Salmonella* spp. and *Vibrio* spp. were found to hybridize with oligonucleotide No. 15 which was obtained from *E. coli* 16S rDNA sequence (92% homology). It means that the phylogenetic distance of these three bacteria genera was much closer than that of the others. Thus, it is necessary to add one or more additional oligonucleotides to the array, depending on the clonal relationship, to allow the discrimination of these bacterial strains.

The variation in the intensity of the hybridization signal may be due to many factors including the extent of sequence homology; the variations of the probe concentrations and the differences in the hybridization stringency for different probes. In addition, cellular protein, lipid, and carbohydrate may mediate significantly nonspecific binding of biotin-labeled probes or variant isolates.

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Phytophthora endoglucanase and a Corresponding Inhibitor Protein

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ABSTRACT

Phytophthora infestans, causal agent of late blight, remains a poorly understood pathogen of potato and tomato. New genotypes or strains arise with increasing virulence in areas where these crops are cultivated. Cultivated varieties of potato and tomato are all susceptible to late blight, but the degree of susceptibility can differ. We have focused on early events in the late blight disease that may contribute to pathogen virulence and levels of host susceptibility. Rapid disintegration of host tissue suggests that cell wall modifying enzymes are an essential factor in disease. Clones for a unique family 12 xyloglucan-specific endoglucanase (XEG) were identified and characterized. The gene encodes a 22 kD extracellular protein that can specifically target the xyloglucan component of the host cell wall. Activity of this enzyme is high during disease progression, as measured by remazol blue-xyloglucan hydrolysis. The gene is present in three copies in the *P. infestans* genome, and provides the first example of a pathogen-encoded family 12 XEG. Family 12 XEGs are known to be inhibited by a specific inhibitor protein. A gene encoding an inhibitor protein that binds specifically to the Phytophthora XEG was identified in potato. The gene is wound responsive and encodes a 43 kD extracellular protein. Overexpression of the XEG inhibitor protein gene from potato, using a Potato Virus X vector, resulted in a marked reduction in the rate of Phytophthora lesion expansion on susceptible potato. Levels of expression of this inhibitor protein may contribute to the reduced susceptibility found in some cultivars and germplasm of potato.

Key words: *Phytophthora infestans*, Late blight, Potato, Tomato, Endoglucanase.

INTRODUCTION

Late blight, caused by *Phytophthora infestans*, continues to be the primary disease in limiting of potato and tomato production. Among the options for control, deployment of resistant germplasm provides the strongest sustainable management practice. Two types of resistance are available, race specific resistance (R) genes, and race-nonspecific genetic mechanisms. While the mechanisms of race non-specific resistance have not been elucidated, resistance has been evaluated as a reduction in rate of lesion expansion. We have initiated studies on the role of host-encoded inhibitor proteins in limiting late blight lesion expansion rates, and the possibility that they contribute to the non-race specific form of resistance. We are specifically investigating proteins that may inhibit extracellular enzymes produced by *Phytophthora infestans*.

Recently a new type of inhibitor was identified from tomato that interacted with a Family 12 xyloglucan-specific endoglucanase. The inhibitor protein, with structural similarity to an inactive form of protease, binds tightly to the Family 12 endoglucanase structure (York et al., 2004) resulting in blockage of the catalytic sites of the endoglucanase. The cellular target of the Family 12 endoglucanases is xyloglucan. The xyloglucans play a critical role in plant cell wall structure by linking the cellulose microfibrils together (Hayashi 1989). It could be expected that xyloglucan hydrolysis would lead to a loosening of the host cell wall structure. This was demonstrated for a Family 12 endoglucanase, where cell wall extension was activated by enzyme treatment (Yuan et al., 2001). Cell wall loosening may facilitate entry by cell wall modifying enzymes and could also lead to membrane instability and water soaking symptoms.

MATERIALS AND METHODS

Identification of family 12 endoglucanase-encoding genes

Family 12 endoglucanases share short regions of conserved amino acid sequence, such as NNLW. Surveying the available EST information for Phytophthora, potential sequences were identified through translational BLAST searches. Genomic DNA and total RNA samples were isolated using DNAeasy and RNAeasy kits from Qiagen. Primers covering the 5′ and 3′ coding regions were used in PCR reactions with PlatinumTaq polymerase, using genomic DNA as a template. Total RNA was also used as a template, following cDNA synthesis with Superscript III Reverse Transcriptase (Invitrogen). Products were cloned into TOPO TA 2.1 and subsequently sequenced.

Total DNA was digested with *Eco* R1 or *Bam* H1, electrophoretically separated and transferred to Hybond nylon membrane. Fluorescein-labelled probe derived from the clone was used to determine copy number in the *P. infestans* genome.

Identification and cloning of the inhibitor protein gene

Using the tomato xyloglucan-specific endoglucanase inhibitor (XIP) gene as a reference (Qin et al., 2003), ESTs were identified from potato using information from GenBank and TIGR databases. Analysis of current database information resulted in an EST that was lacking two nucleotides at the 5′end, which after incorporation, provided the full coding sequence. Forward and reverse primers were added to a PCR reaction mix and amplified with PlatinumTaq polymerase (Invitrogen). The single product obtained was cloned into the TOPO TA cloning vector 2.1 and the cloned product was sequenced.

Over-expression of XIP.

To test the role of the inhibitor protein on infection and spread of *Phytophthora infestans*, the coding region for XIP was introduced into a Potato virus X vector (Faivre et al., 2004). The XIP gene was amplified with primers containing *Nru 1* and *Sal 1* sites and recloned into TOPO TA 2.1 vector. Insert was removed by digestion and

placed into the PVX vector. Linearized PVX vector was used to generate infectious transcripts for inoculation onto *Nicotiana benthamiana*. After propagation for one week, virus was transferred from tobacco to six week old potato (*Solanum tuberosum* cvs. Kennebec and Green Mountain) by mechanical inoculation. Plants were maintained in greenhouse conditions for two weeks. Expression of the XIP gene from PVX was monitored by reverse transcriptase-PCR using primers that spanned the region at the junction of the PVX vector and the XIP gene.

Disease evaluation on detached leaflets

Fourteen days after inoculation, systemically infected leaves were excised and placed abaxial side up on moist paper towels. Leaves were inoculated with a sporangial suspension of *Phytophthora infestans* US race 8, and incubated in enclosed containers. Disease symptoms were evaluated visually at 5 and 8 days post-inoculation. Immature tubers were harvested and inoculated in the same manner as the detached leaves.

Assay for xyloglucan-specific endoglucanase activity

Suspensions of Remazol Brilliant Blue linked xyloglucan (Megazyme) were prepared as a 0.1% w/v mixture in water. Samples (0.5 ml) were aliquoted into microfuge tubes, sealed and stored at room temperature. Tissue samples were extracted with three volumes of water, microfuged and 0.1 ml samples of supernatant transferred to tubes containing labeled xyloglucan.. Incubations were carried out overnight at room temperature. Samples were then precipitated with equal volumes of ethanol, and microfuged for five minutes. Xyloglucanase activity was observed as a reduction in pellet size and increase in soluble blue dye.

RESULTS

Identification of family 12 homologs in *Phytophthora infestans*.

Performing BLAST searches using the conserved domains from Family 12 protein allowed for identification of a Family 12 endoglucanase in *P. infestans*. The gene encodes an acidic protein of about 24 kD (Figure 1a). As expected, the gene lacks introns in the genomic coding sequence. Comparison of the *Phytophthora* sequence showed 69% amino acid conservation with an *Aspergillus nidulans* homolog. Southern analysis revealed at least three copies in four isolates analyzed. Homologs of the Family 12 xyloglucan-specific endoglucanase are also present in other *Phytophthora* species.

Identification of a potato encoded XIP.

Clones were obtained from genomic DNA by PCR amplification and shown to be identical to the EST sequences, indicating a lack of introns. The gene encodes a basic protein of about 48 kD (Fig. 1b.). The clone showed 98% amino acid conservation with the tomato homolog, 80 % conservation with an extracellular dermal protein from carrot (Satoh et al., 1992), and 78% conservation to the same protein in Arabidopsis. In carrot, inhibitor production was up-regulated by wounding and stress, suggesting an active role in plant defense.

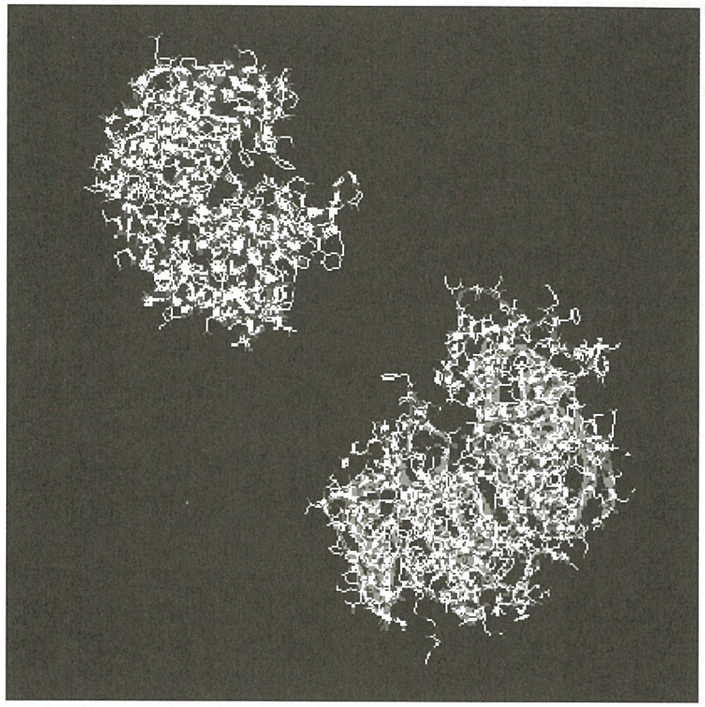

Fig. 1. Models of egl12 (left) and XIP (right) developed with Swissmodel.

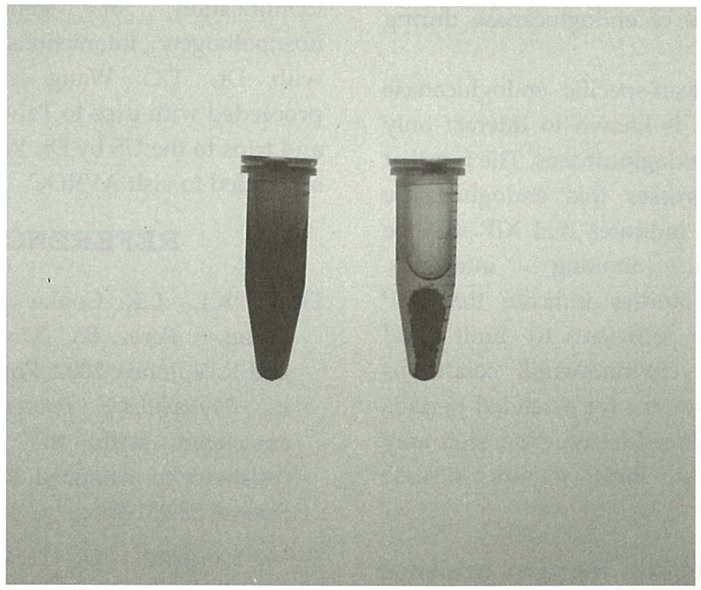

Fig. 2. Xyloglucanase activity measured by remazol brilliant blue xyloglucan.

Over-expression analysis with PVX vector

Expression of the inhibitor protein resulted in a 25-35% reduction in water-soaked lesion area five days after inoculation. Continued reduction was seen at eight days post-inoculation. Tuber inoculations also resulted in lower tissue damage in the presence of PVX-expressed inhibitor protein. Tuber infection symptoms were different. Tubers with enhanced inhibitor protein were firm and darkened, while the normal infections were not discolored, but water-soaked. Assays for xyloglucan-specific endoglucanase activity, using Remazol dyed xyloglucan revealed strong activity in infected tissues (Fig. 2).

To date, no Family 12 endoglucanases have been identified in *Phytophthora*, although other endoglucanases have been identified (McLeod et al 2003). This study also provides the first evidence that any phytopathogenic micro-organisms actively express this type of endoglucanase during infections.

The xyloglucan-specific endoglucanase inhibitor protein is known to interact only with Family 12 endoglucanases. The fact that *P. infestans* expresses this endoglucanase during infection indicates that XIP may be important in limiting infections. Overexpression studies indicate that XIP levels may be sufficient to limit field infection where environmental conditions are not often favorable for extended periods of time, thus reduced lesion expansion may be sufficient to limit overall disease significantly.

This study provides the first evidence that xyloglucan-specific endoglucanase inhibitor protein can play a role in late blight disease development

Differences in steady state or induced levels of this protein in various cultivars and germplasm may contribute to durable, non-R gene mediated resistance. Further work will study various cultivars and germplasm to determine if a correlation exists between the levels of XIP present and the level of resistance to late blight and other diseases. Further cloning and screening of sequences from *P. infestans* will be needed to determine the expression level of each copy of family 12 endoglucanase, and determine if a correlation exists between expression levels and pathogen virulence (Deahl *et al.*, 2002).

Exchange of Scientific Information and Technology

Our laboratory and Dr. Deahl's laboratory work together on issues related to molecular characterization of *P. infestans*. Emphasis has been on molecular identification of pathogen populations and identification of genes regulating host:pathogen interactions. Collaboration with Dr. T.C. Wang at AVRDC has proceeded with trips to Taiwan by Dr. Deahl and trips to the US by Dr. Wang. Dr. Jones is scheduled to visit AVRDC.

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Monitoring *Phytophthora infestans* in Taiwan for Population Shifts that May Affect Late Blight Management Strategies

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ABSTRACT

Late blight caused by *Phytophthora infestans* is one of the most destructive diseases on tomato in Taiwan, especially in the eastern Taiwan. A total of 307 isolates of the pathogen collected by AVRDC- The World Vegetable Center during 2003 to 2005 were aggressive to both tomato and potato. All isolates were A1 mating type and all, except one isolate collected in 2003, were resistant to Metalaxyl. They were classified into ten physiological races by using six tomato differentials. All the isolates were analyzed by molecular genotype characterization. Most isolates (303/307) had dilocus allozyme genotypes 100/111 and 100/100, except 3 isolates were 100/122 and 100/100, for the loci coding the glucose-6-phosphate isozyme (*Gpi*) and peptidase (*Pep*), respectively. Mitochondrial haplotype analyses of polymorphic regions of the mitochondrial genome (mtDNA) were IIb. These characters, together with RG57 fingerprinting, indicated that these isolates belonged to the US-11 clonal lineage, containing 25% variant. There was only one isolates analyzed as 86/100 and 100/100 allozyme genotype for *Gpi* and *Pep*, Ib mtDNA haplotype, and belong to US-1 clonal lineage with RG57 fingerprinting. The results indicated that no new genotype of *P. infestans* immigrated into Taiwan during 2003-2005. Under the travel funding supported from Research and Scientific Exchanges Division, International Cooperation and Development, USDA, The principal leader Dr. T. C. Wang in AVRDC and the project collaborator, Dr. K. L. Deahl in the USDA-ARS Vegetable Lab hold meetings in Taiwan and USA for exchanging new scientific information and technology, setting up the laboratory procedures in AVRDC, and planning further collaboration. Dr. Deahl and his assistant Frances Perez also provided the kind assistance to hold a training workshop for molecular characterization of *P. infestans* isolates for AVRDC's and Taiwan researchers in 2004. Increasing percentage of the US-11 variant indicated that genomic diversity of the pathogen are changing quickly although sexual recombination was not yet detected. Therefore, it is important to monitor the population change of the *P. infestans* and develop the integrated management strategy for the disease control continuously.

Key words: *Phytophthora infestans*, Physiological race, Mating type, Metalaxyl, Genotype characterization

INTRODUCTION

Late blight, caused by *Phytophthora infestans* (Mont.) de Bary, is a highly destructive disease affecting both tomatoes and potatoes. The pathogen is most active during cool, moist weather conditions. The disease has increased in severity in many parts of the world in recent years. This has been associated with the migration of new and more aggressive populations of the pathogens (Spielman *et al.*, 1991). Since 1998 severe late blight epidemics have occurred in Taiwan both on tomato and potato crops concomitant with the appearance of a new genotype US-11, of the pathogen (Ann and Chang, 2000). It is highly plausible that the new genotype was introduced on imported table potatoes during 1997-98 growing season (AVRDC, 1999; Ann and Chang, 2000; Deahl *et al.*, 2002). By 2000 the new genotype had almost completely displaced the old US-1 genotype, which all Taiwan isolates collected during 1991-97 (Deahl *et al.*, 2002;). The new US-11 genotype appears to be more aggressive on tomato and potato. Moreover it carries resistance to metalaxyl, a fungicide on which Taiwan tomato farmers had relied for several years to manage the disease (AVRDC, 1999; Ann and Chang, 2000). Fortunately, US-11 is of the A1 mating type as was the old US-1 genotype. And the A2 mating type of *P. infestans* has not yet migrated into Taiwan. The introduction of the A2 mating would increase genetic recombination through sexual reproduction locally, and has been a concern for local quarantine.

AVRDC has been breeding for tomato late blight resistance since 1994 utilizing resistant sources from *Lycopersicon pimpinellifolium*, accession L3708, and *L. hirsutum*, accession LA1033, which were selected for resistance against the old genotype US-1 (AVRDC, 2000; Chungwongse *et al.*, 2002). Thus far, these resistance sources also hold up against the new genotype US-11 of *P. infestans* until 2004. However, the resistance of AVRDC's resistant lines was broke-down due to the occurrence of new races (Race T1, 3, & T1, 2, 3, 4) during 2003 (AVRDC, 2004). Therefore, there is a need to constantly monitor the population for possible emergence of new physiological races and for the introduction of additional genotypes, in particular those that may be of the A2 mating type. Knowledge of this kind is essential to develop appropriate breeding approaches and the overall disease management strategies.

This project was designed to assess occurrence of and severity of late blight island-wide in tomato and potato crops through seasonal survey in Taiwan during 2003 to 2005, and to monitor for changes in the late blight pathogen, *P. infestans* for the management strategies of late blight control and for the development of quarantine regulations by the Bureau of Animal and Plant Health Inspection and Quarantine, Council of Agriculture. This project was also planned to invite Dr. Kenneth L. Deahl, Plant Pathologist in USDA-ARS Vegetable Lab. at Beltsville, and his assistant Ms. Frances Perez to visit Taiwan and AVRDC–The World Vegetable Center to present seminars for discussing and exchanging current information on local and international dissemination of new isolates of *P. infestans*

with Taiwan scientists and to be resource persons for the training workshop of "molecular genotype characterization on *P. infestans*" at AVRDC for transferring the advanced molecular bio-technology with the researchers in universities and research institutes.

MATERIALS AND METHODS

Island-Wide Surveys on Late Blight of Tomato and Potato Crops in Taiwan, and Pathogen Isolation

Island-wide surveys on late blight of tomato and potato crops were made in most counties in Taiwan to assess late blight occurrence, severity, and to collect infected host tissues. Pathogen isolates were isolated from diseased samples by cutting out host tissue pieces (3-4mm^2) from the margins of lesions, surface sterilizing, and transferring to petri plates containing rye A agar amended with antibiotics, including 100 ppm ampicillin, 100 ppm nystatin, and 50 ppm rifampicin (Caten and Jinks, 1968). Pure isolates were preserved on autoclaved rye grain with water at 16-20°C.

Physiological Race Identification

A total of 158 isolates were artificially inoculated with zoospore/sporangia suspension (5 x 10^4/ml) on six tomato differentials: TS19 (*Ph*$^+$); TS33 (*Ph*-1); W.Va.700 (*Ph*-1, 2); CLN2037B (*Ph*-1, 2, 3); L3708 (*Ph*-1, 2, 3, 4), and LA1033 (*Ph*-1, 2, 3, 4, 5) for the first 24 hr at 100% RH and 20±2°C without light, thereafter, maintained at 60-95% RH and 20±2°C with a 14-hr light (70μE.m^{-2}.5^{-1}) period per day. The pathogenicity of each isolates was evaluated 7 and 10 days after inoculation (AVRDC, 1999). Based on the mean disease severity rating of differentials, isolates could be grouped (Vartanian and Endo, 1985; AVRDC, 1999). The designations of these races are derived from the genes the races defeat.

Host Specialization

All isolates were tested for their aggressiveness to potato by inoculating "Kennebec" potato plants at 49 days after planting. Susceptible tomato variety FMTT-22 was arranged as the control. The inoculation method, incubation conditions, and disease severity rating were the same as above-mentioned.

Assessment of Metalaxyl Sensitivity *in Vitro*

Metalaxyl sensitivity of isolates was determined by measuring colony radial diameter twice at 90° angle for each isolate colony size after cultured for 14 days at 20°C on rye A agar medium amended with 100ppm of metalaxyl (Pappas, 1985). Two measurements were averaged as colony diameter. Three replications of each isolate were averaged for the analysis by Duncan's Multiple Range Test (P≤0.05) for the comparison. Sensitive isolates, Pi39, 43, 64 and 75 were arranged as the control.

Determination of Mating Type

Mating type of *P. infestans* isolates was determined by pairing each isolate with known A1 (isolate P991) and A2 (isolate P731) mating type standard cultures of *P. parasitica* on rye A agar; paired cultures were incubated at 20±1℃ in the dark for 10-14 days. Isolate that formed oospores/ oogonia when paired with known A2 mating type were designed A1 mating types; those forming the sexual reproductive structures with known A1 mating type were designed as A2 mating type (Hohl and Iselin, 1984).

Molecular Genotype Characterization of *P. infestans*

Molecular genotype characterization was followed by the global marker database (Forbes *et al.*, 1998). Genotypes at two polymorphic allozyme loci, *Gpi* and *Pep*, were determined using the protocols developed by Goodwin *et al.* (1995). Enzymatic activities were revealed following electrophoresis and staining with the appropriate agar overlays. The mtDNA haplotypes of isolates were determined by PCR-RFLP using a method modified from Griffith and Shaw's protocol (1998). P2 and P4 DNA were amplified by oligonucleotide primers, F2/R2 and F4/R4, and then restricted by *Msp*I and *Eco*RI, respectively. Digested DNA patterns were resolved by electrophoresis run at approximately 10V cm^{-1} for 1-1.5 hours and visualized using a UV transilluminator at 254 nm. DNA fingerprinting using the moderately repetitive probe RG57 was carried out as described by Goodwin *et al.* (1998). The genomic DNA of *P. infestans* was transferred to Hybond N+ Nylon membrane and then hybridized by the RG57 probe, labeled with fluorescein. All of the new isolates were also sent to USDA-ARS Vegetable Lab at Beltsville for molecular genotype characterization for the comparison with the isolates collected from other countries in the world.

Exchange of Scientific Information and Technology

Under the travel funding supported from Research and Scientific Exchanges Division, International Cooperation and Development, USDA, the project was designed to hold meetings in both Taiwan and USA for exchanging new scientific information and technology, setting up the laboratory procedures in AVRDC and planning further collaboration. AVRDC and Dr. Deahl also cooperated to hold a training workshop for molecular characterization of *P. infestans* for the research staffs at AVRDC and local research institutes.

RESULTS

Island-Wide Surveys on Late Blight of Tomato and Potato Crops in Taiwan and Pathogen Isolation

Late blight was continually occurred, devastated and caused severe economic losses in highland production areas and eastern Taiwan during spring season due to lower night temperature and higher relative humidity. A total of 307 isolates of *P. infestans* were isolated from diseased tomato and potato samples collected from 14 counties in Taiwan.

Identifications of Physiological Races, Host Specialization, Metalaxyl Sensitivity, and Mating Type

All the isolates were A1 mating type, resistant to metalaxyl (except one collected in 2003), and aggressive on both tomato and potato without host specificity. A total of 158 isolates collected in 2004 and 2005 were grouped into ten physiological races consisting of: Race T1; T1,2; T1,3; T1,2,3; T1,2,5; T1,3,5; T1,2,3,4; T1,2,3,5; T1,3,4,5; and T1,2,3,4,5 (Table 1). Race T1,2,3 seems the predominant one.

Molecular Genotype Characterization of *P. infestans*

Most isolates (303/307) had dilocus allozyme genotypes 100/111 and 100/100, except 3 isolates were 100/122 and 100/100,

for the loci coding the glucose-6-phosphate isozyme (*Gpi*) and peptidase (*Pep*), respectively. Mitochondrial haplotypes evaluated through PCR amplification and RFLP analysis of polymorphic regions of the mitochondrial genome (mtDNA) were IIb mtDNA haplotype. These characters, together with RG57 fingerprinting, indicated that these isolates belonged to the US-11 clonial lineage, containing 25% (77/306) variant. There was only one isolates analyzed as 86/100 and 100/100 allozyme genotype for *Gpi* and *Pep*, Ib mtDNA haplotype, and belong to US-1 clonal lineage with RG57 fingerprinting (Table 2).

Exchange of Scientific Information and Technology

The principal leader, Dr. Tien-cheng Wang and Assistant Mr. Zong-ming Sheu were invited by project collaborator, Dr. K. L. Deahl, to visit USDA-ARS Vegetable Lab at Beltsville, MD, USA during September 12th to 25th in 2003 to exchange information of population shift of *P. infestans* in the world and to learn the molecular genotype characterization technology; AVRDC's pathologist Dr. J. F. Wang met with Dr.

Table 1. Designation of putative physiological races of *Phytophthora infestans* isolates collected from tomato in Taiwan from 2004 to 2005.

Race	TS19 (*Ph*+)	TS33 (*Ph*-1)	W. Va. 700 (*Ph*-1,2)	CLN2037B (*Ph*-1,2,3)	L3708 (*Ph*-1,2,3,4)[1]	L1033 (*Ph*-1,2,3,4,5)[2]	No. of Isolates
T1	S	S	R	R	R	R	13
T1,2	S	S	S	R	R	R	20
T1,3	S	S	R	S	R	R	14
T1,2,3	S	S	S	S	R	R	66
T1,2,5	S	S	S	R	R	S	1
T1,3,5	S	S	R	S	R	S	2
T1,2,3,4	S	S	S	S	S	R	16
T1,2,3,5	S	S	S	S	R	S	17
T1,3,4,5	S	S	R	S	S	S	2
T1,2,3,4,5	S	S	S	S	S	S	7

[1,2]Gene(s) conditioning late blight resistance in this accession are not yet characterized.

Table 2. The genotype characteristics of *Phytophthora infestans* isolates collected in Taiwan during 2003 to 2005.

No. of isolates collected	RG57[1]			Gpi/Pep[3]			mtDNA haplotype[4]	
	US-1	US-11	US-11v[2]	86/100 92/100	100/111 100/100	100/122 100/100	Ib	IIb
307	1	229	77	1	303	3	1	306

[1]DNA fingerprint analysis using RG57 probe.
[2]Variant that misses one band (band 5 or 10) and/ or appears one band (band 12 or 18) on fingerprinting pattern.
[3]Allozyme genotype analysis.
[4]Mitochondrial haplotyping through PCR and RFLP analysis.

Deahl in the American Phytopathology Society 2004 Annual Meeting hold at Anaheim, California during August 31st to September 1st to exchange the recent research results and discuss the international dissemination information of *P. infestans*. Furthermore, they also confirmed the detail of the train workshop that would be hold at AVRDC in October 2004.

Project collaborator Dr. Deahl, Assistant Ms. Frances Perez, and former AVRDC plant pathologist Dr. Lowell L. Black were invited to visit AVRDC and Taiwan by Dr. Thomas A. Lumpkin, Director General of AVRDC during October 21st to 30th in 2004. They visited Nantou County on October 24 and observed destructive late blight in many tomato fields. Dr. Deahl presented the seminar entitled "Population changes in *Phytophthora infestans* in the USA and Canada associated with the appearance of resistance to metalaxyl and the A2 mating type" at AVRDC on October 25. Dr. Deahl was also invited as a resource person of the training workshop "Molecular genotype Characterization" hold by AVRDC during October 25th to 27th. Dr. Deahl and Ms. Frances advised AVRDC on setting up laboratory procedures for molecular genotype characterization of the isolates during the workshop. A total of 14 participants from local institutes, universities and AVRDC attended the training workshop. Dr. Deahl and Dr. Black visited AVRDC's scientists to understand their on-going research activities and also to discuss the possibility of future collaboration.

Drs. Deahl, Black and Ms. Frances accompanied with Dr. T.C. Wang visited Department of Plant Pathology, National ChungHsin University, and Department of Microbiology and Plant Pathology, National Taiwan University to present the seminars on October 28th and October 29th, respectively. They reviewed with Taiwan professors, scientists and graduate students on methodologies for characterizing *P. infestans* isolates and exchanged the information of advanced biotechnology. Their visit improved the communication of new message and the exchange of advanced biotechniques. They also visited Bureau of Animal and Plant Health Inspection Quarantine (BAPHIQ) to meet with senior officers Drs. Huang, Tze-Chung and Kuo, Ker-cheng for exchanging the experience on recent international dissemination of *P. infestans* and quarantine regulations, which could be valuable for the development of quarantine strategies.

Mr. C. H. Chen was invited and visited to USDA-ARS Vegetable Lab during November 15th to 25th in 2005, to update techniques on molecular characterization, especially the technique of rapid detection A2 mating type of *P. infestans*, which is very helpful for the quarantine.

Dr. T. C. Wang and Assistant Z. M. Sheu and C. H. Chen attended the American Phytopathology Society (APS) 2005 Annual Meeting in Austin, USA, during July 30th to August 3rd, presented a paper related to "The variation of race composition of *Phytophthora infestans* in Taiwan during 1991 to 2004", and exchanged information with scientists in APS. Then they visited Dr. K. L. Deahl and his post-doctor Dr. Stefano Costanzo at USDA-ARS Vegetable Lab. in Beltsville during August 4th to 8th to update molecular genotype characterization techniques, discuss on the project results, and plan for future collaboration.

CONCLUSIONS

Late blight was epidemic and caused severe yield loss in the eastern Taiwan as well as in highland areas where were lower temperature and higher relative humidity in the growth season of tomato and potato during 2003-2005. All isolates collected in this study were very aggressive and resistant to metalaxyl. That maybe why the farmers were difficult to get effective disease control by fungicides application. The development of an integrated disease management strategy will be very important and urgent.

Ten physiological races of *P. infestans* were identification from 130 isolates. Race T1,2,3 seems the predominant race during 2004 to 2005. It is critical, therefore, to continue monitor the occurrence of new races of *P. infestans* in the future.

The results of the mating type identification also indicated that the A2 mating type of *P. infestans* has not yet found into Taiwan. If the A2 mating type was migrated into Taiwan, it will become a complex problem due to its sexual reproduction. Mating type is very important to the quarantine regulations of BAPHIQ, the rapid detection of mating type will be useful to improve the quarantine efficiency.

The results of the molecular genotype characterization showed that new genotype US-11 has almost completely displaced the old population US-1. No new genotypes were identified during 2003 to 2005. However, increasing percentage of the US-11 variant indicated that genomic diversity of the pathogen is changing quickly although sexual recombination was not yet detected. Therefore, it is necessary to monitor the population shifts of the *P. infestans* and develop the integrated management strategy for the disease control continuously.

This successful collaboration activity not only documented the population shifts of *Phytophthora infestans* in Taiwan for the important references of BAPHIQ to develop the quarantine strategy but also improved the communication of new message and the exchange of advanced biotechniques between AVRDC and USDA-ARS Vegetable Lab. This will be a good example for future collaboration.

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